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This is a request for filing a
 X new utility patent application under 37 CFR 1.53(b)(1).
 _____ continuation-in-part under CFR 1.53(b)(2) of prior
 application serial no. _____, filed _____
 (list entire parentage).

Title: SOMATIC TRANSGENE IMMUNIZATION AND RELATED METHODS

Inventor(s) (full name of each inventor): **MAURIZIO ZANETTI**

Enclosed are:

 X Return receipt postcard
 Initial Information Data Sheet
 X 1 Page application cover sheet
 X 135 Pages of specification (includes claims and abstract)
 X 37 Sheets of drawing(s)
 Pages of an executed Declaration for Patent Application
 An executed Power of Attorney for Patent Application by
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 Statement Under 37 CFR 1.821(f)
 An executed assignment and cover sheet
 An executed small entity statement
 Also enclosed:

Inventor: Maurizio Zanetti
Docket No.: P-ZA 3519
Page 2

___ This application is based on prior foreign application(s)
No.(s) _____, filed in _____ on _____
_____, respectively, and priority is hereby claimed therefrom.

X This application is based on, and claims the benefit of, U.S.
Provisional Application No. 60/083,154, filed April 27, 1998,
and entitled **SOMATIC TRANSGENE IMMUNIZATION AND RELATED
METHODS**.

___ This application is based on, and claims the benefit of, U.S.
Provisional Application No. 60/_____ filed _____, which
was converted from U.S. Serial No. _____, and entitled
_____.

The filing fee has been calculated as shown below:

	Number Filed		Number Extra		Rate			Fee	
					Small Entity	Other Entity		Small Entity	Other Entity
Total Claims	33-20	=	13	x	\$9	\$18	=	\$	\$
Indepen- dent Claims	5-3	=	2	x	\$39	\$78	=	\$	\$
Multiple Dependent Claims Presented:___ Yes <u>X</u> No					\$130	\$260		\$	\$
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Date: April 27, 1999

Respectfully submitted,

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A P P L I C A T I O N

for

UNITED STATES LETTERS PATENT

on

SOMATIC TRANSGENE IMMUNIZATION AND RELATED METHODS

by

Maurizio Zanetti

Sheets of Drawings: 37

Docket No.: P-ZA 3519

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SOMATIC TRANSGENE IMMUNIZATION AND RELATED METHODS

This application claims the benefit of priority
of provisional application serial No. 60/083,154, filed
5 April 27, 1998, the entire contents of which is
incorporated herein by reference.

This invention was made in part with government
support under grant number PO1 AI33204 and AI36467,
10 awarded by the National Institutes of Health. The
government has certain rights in the invention.

BACKGROUND OF THE INVENTION

15 In recent years, there has been an explosion of
information on the new possibility of inducing immunity
using nucleic acids, called DNA vaccination. Previous
studies have shown that plasmid DNA introduced into an
adult immunocompetent host could induce an antibody
20 response (Tang et al., Nature 356:152-154 (1992)), it was
soon demonstrated using the influenza virus that both
humoral and cell-mediated could be induced, and these
were sufficient for protection *in vivo* (Ulmer et al.,
Science 259:1745-1749 (1993); Fynan et al., Proc. Natl.
25 Acad. Sci. USA 90:11478-11482 (1993)). It soon became
evident that, DNA vaccines, also called genetic vaccines,
have gone through a phase of exponential growth and found
an application against a large variety of antigens. They
have been applied to immunize against cancer (Conry et
30 al., Cancer Res. 54:1164-1168 (1994); bacteria (Tascon et
al., Nat. Med. 2:888-892 (1996); Huygen et al., Nat. Med.
2:893-898 (1996)); virus (Ulmer et al., *supra*, 1993;
Fynan et al., *supra*, 1993; Raz et al., Proc. Natl. Acad.
Sci. USA 91:9519-9523 (1994); Davis et al., Vaccine
35 12:1503-1509 (1994); Wang et al., Proc. Natl. Acad. Sci.

USA 90:4156-4160 (1993); and parasites (Sedegah et al.,
Proc. Natl. Acad. Sci. USA 91:9866-9870(1994)).

This basis of DNA vaccination is the
 5 introduction into a host of the "blue-print" for vaccine
 molecules in a way that mimics viral infections without
 the infectious threat. The use of DNA vaccines can be of
 particular value in those instances in which vaccines are
 either ineffective or not yet available.

10 Genetic vaccines, while conceptually simple in
 their making, for example, a bacterial plasmid
 incorporating the coding region of interest, they are
 still fraught with a number of unresolved issues
 15 pertaining mainly to their biology at the site of
 injection and systemically. Genetic vaccines work along
 the same lines of recognition of antigen (B cells) and
 antigen processing and presentation (T cells) known and
 established for protein antigens or infectious pathogens.

20 It has been known for many years that
 eukaryotic DNA is per se scarcely immunogenic whereas
 prokaryotic (bacterial) DNA possesses properties relevant
 to immunogenicity. It has been known for some time that
 25 bacterial DNA could serve as adjuvant in immunization
 (Braun et al., Proc. Soc. Exp. Biol. Med. 119:701
 (1965)). It was subsequently reported that mice
 immunized with *Escherichia coli* DNA complexed with
 methylated BSA in adjuvant produce significantly greater
 30 amounts of antibodies than mice immunized with calf
 thymus DNA (Gilkeson et al., Clin. Immunol. Immunopathol.
 51:362-371 (1989); Gilkeson et al., J. Immunol. 142:1482-
 1486 (1989)). Earlier observations had already shown
 that bacterial DNA possesses immunostimulatory properties
 35 (Tokunaga et al., J. Natl. Cancer Inst. 72:955-962

(1984), a fact undisputed today (Messina et al., Cell Immunol. 147:148-157 (1993)). These stimulatory properties are apparently linked to a six-base nucleotide motif consisting of an unmethylated CpG dinucleotide (Krieg et al., Nature 374:546-549 (1995) expressed nearly twenty times more frequently in bacterial than in vertebrate DNA (Cardon et al., Proc. Natl. Acad. Sci. USA 91:3799-803 (1994)). Noncoding, immunostimulatory sequences (ISS)-enriched plasmid DNA or ISS oligonucleotides themselves stimulate immune responses to co-administered antigens (Roman et al., Nat. Med. 3:849-854 (1997)) by activating IFN-g, IL-12 and IL-18, all of which promote a Th1 response (Carson and Raz, J. Exp. Med. 186:1621-1622 (1997)).

In 1992, plasmid DNA was shown to immunize against the very antigen it codes for (Tang et al., Nature 356:152-154 (1992)). Therefore, it was possible to immunize using foreign DNA controlled by a potent promoter for tissue expression. Inoculation of functional genes into somatic cells of adult immunocompetent animals is a simple way to mimic natural infection and initiate adaptive immunity (Ulmer et al., Curr. Opin. Immunol. 8:531-536 (1996)). Plasmid DNA containing antigen-coding sequences and regulatory elements for their expression can be introduced in tissues by parenteral injection (Wang et al., *supra*, 1993) or by particle bombardment (Tang et al., *supra*, 1993). Antibody production (B-cell immunity), and cell-mediated immunity of the helper or cytotoxic T cell-type, have been induced against viruses (Ulmer et al., *supra*, 1993), bacteria (Huygen et al., *supra*, 1996; Tascon et al., *supra*, 1996), parasites (Sedegah et al., *supra*, 1994), tumor antigens (Conry et al., *supra*, 1994), self antigens (Gilkeson et al., J. Exp. Med. 183:1389-1397

(1996); Waisman et al., Nat. Med. 2:899-905 (1996)) and allergens (Raz et al., Proc. Natl. Acad. Sci. USA 93:5141-5145 (1996)).

Typically, injections of plasmid DNA via the intramuscular or intradermal route yields both antibody and cellular responses with long-lasting immunity preferably induced by multiple DNA inoculations (Sedegah et al., *supra*, 1994; Xiang et al., *supra*, 1994). The transgene product is, however, rarely found in the circulation (Davis et al., *supra*, 1993), and little is known about where and how antigen presentation occurs.

From a practical stand-point, immunization via DNA inoculation relies on *in vivo* transfection, production and possibly secretion of the transgene product, and antigen presentation by specialized cells. However, in most studies, neither the *in vivo* transfected cells nor the antigen presenting cells involved in this process have been identified. Expression of foreign DNA under the control of viral promoters (Tang et al., *supra*, 1992; Ulmer et al., *supra*, 1993; Davis et al., *supra*, 1993; Raz et al., *supra*, 1994; Wang et al., *supra*, 1993; Huygen et al., *supra*, 1996; Tascon et al., *supra*, 1996; Sedegah et al., *supra*, 1994; Dollan et al., *supra*, 1996) limit tissue specificity. Therefore, no control of expression is possible other than the site of DNA inoculation.

Although genetic vaccines have been used successfully, there remains a need to develop more effective methods to exploit the immunogenic potential of genetic vaccines. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The invention provides a method for stimulating an immune response by administering to a lymphoid tissue a nucleic acid molecule comprising an expression element operationally linked to a nucleic acid sequence encoding one or more heterologous epitopes. The heterologous epitope can be inserted into a complementarity-determining region of an immunoglobulin molecule. The invention also provides a nucleic acid molecule comprising a hematopoietic expression element operationally linked to a nucleic acid sequence encoding a heterologous polypeptide. The invention also provides a method of treating a condition by administering a nucleic acid molecule comprising a hematopoietic cell expression element operationally linked to a nucleic acid sequence encoding a heterologous polypeptide, wherein the nucleic acid molecule is targeted to a hematopoietic cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of plasmid DNA γ 1WT and its γ 1WT-TAC and γ 1NANP variants. The γ 1WT H chain construct is the product of the fusion between a human γ 1 constant (C) region gene present in the plasmid vector pNeoy1 with the murine V_H^{62} gene (2.3 kb) (Sollazzo et al., *supra*, 1989). The V_H region gene is productively rearranged and the C region gene is in genomic configuration. Variants γ 1WT-TAC and γ 1NANP contain the nucleotide insertions shown in bold characters in CDR3. Each plasmid DNA carries the regulatory elements, promoter (Pr) and enhancer (En) needed for tissue-specific expression. In plasmid DNA γ 1NANP the human γ 1 C region gene is joined to a

productively rearranged murine variable (V) region gene modified in the third complementarity determining region (CDR3) by introduction of the nucleotide sequence coding for three Asn-Ala-Asn-Pro repeats. In these plasmids, the promoter and enhancer elements are those constitutively existing in Ig H chain genes. Neo^r=neomycin resistance gene; Amp^r=ampicillin resistance gene; PR=promoter; EN=enhancer; C_H=heavy chain C region; V_H=heavy chain variable region; FR=framework region; CDR=complementarity determining region.

Figure 2 shows a kinetic analysis of the anti-immunoglobulin response and immunoglobulin heavy (H)-chain transgene immunoglobulin production in mice inoculated intraspleen with plasmid DNA γ 1WT. Panel A shows antibody response. Each column corresponds to a group of six individual C57Bl/6 mice. All mice were inoculated at the same time. They were bled at refracted time intervals to separate two consecutive bleedings on the same animal in compliance with regulations for humane animal care. Therefore, an identical pattern identifies the same group of mice. The columns referring to day 57 and 80 are the mean value of mice from all three groups (18 mice). Values represent means \pm SD of absorbances \pm (A_{492}) obtained by ELISA. Tests were done in duplicate at 1:1,600 dilution. Panel B shows the production of transgenic immunoglobulin. Each symbol corresponds to individual determinations (single mouse) at the time indicated on the abscissa. Groups of individual C57Bl/6 mice were inoculated at the same time. Mice were bled at refracted time intervals to separate two consecutive bleedings on the same animal in compliance with regulations for humane animal care. Values represent the absorbance (A_{492}) obtained by a capture ELISA as detailed in the Material and Experimental Procedures. Symbols

define single mice from the same group of mice. Tests were done on sera diluted 1:10.

Figure 3 shows the effect of different routes of inoculation on the primary antibody response against the γ 1NANP immunoglobulin following plasmid DNA injection. Each group is composed of four mice. Antibody titers (Log_{10}) were determined by ELISA. Data shown represent the mean of individual titers which were determined on the basis of the last value with an absorbance \geq of 0.200 (A_{492}).

Figure 4 shows studies on transgene transcription. Panel A shows semiquantitative analysis of the human γ 1 C (466 bp) and murine V_H^{62} (198 bp) region genes at sequential times (0, 10, 20 and 127 days) after intraspleen DNA inoculation. cDNA was reverse-transcribed from mRNA, extracted from equal amounts (11 mg) of frozen spleen tissue, and used as a template DNA for further amplification by PCR. β -actin DNA served as a control for mRNA content. Panel B shows Southern blot analysis of the PCR-amplified product from spleen of animals inoculated with plasmid DNA γ 1WT and harvested at successive times (0, 10, 20 and 127 days) after intraspleen DNA inoculation. The PCR product was probed with a specific synthetic oligonucleotide for the human γ 1 C region probe. Lane 1 shows the 466 bp gene segment of the human γ 1 C region amplified from the plasmid pNeo γ 1, which was used as a positive control. Lane 2 shows the amplified 198 bp murine V_H^{62} gene used as negative control; lane 3 - 6: DNA amplified with human γ 1 C region-specific primers at different times (0, 10, 20 and 127 days) after intraspleen DNA inoculation. Panel C shows Southern blot analysis of the PCR-amplified V_H product (240 bp) from the spleen of a mouse inoculated

with γ 1NANP DNA and harvested on day 15. The PCR product was probed with a specific synthetic oligonucleotide for the NANP coding sequence in CDR3. Lane 1 shows DNA from a naive mouse. Lane 2 shows cDNA amplified with murine V_H region-specific primers. Lane 3 shows plasmid DNA γ 1NANP (positive control).

Figure 5 shows the nucleotide sequence of genomic DNA clones corresponding to the productively rearranged VDJ region of γ 1WT-TAC DNA. A 520 bp fragment was amplified from (1) genomic DNA extracted from a spleen inoculated 17 days earlier with plasmid DNA γ 1WT-TAC, and (2) J558L cells constitutively harboring plasmid DNA γ 1WT (Sollazzo et al., *supra*, 1989). The amplified products were cloned and sequenced using two different primers from opposite directions. The top nucleotide sequence refers to γ 1WT-TAC and serves as a reference. SP7-SP12 identify six clones originated from splenic genomic DNA. TR35-TR38 identify four genomic DNA clones derived from transfectoma cells. The CDR and framework regions (FR) are indicated. This study indicates that after injection *in vivo* the transgene does not undergo somatic mutation.

Figure 6 shows anti-immunoglobulin antibodies produced by C57Bl/6 mice inoculated intraspleen with plasmid DNA γ 1WT react with the V region. Panel A shows representation of the engineered antibodies used to study the ELISA reactivity. V_H^{62} and V_H^{36-65} are two unrelated murine V_H region gene products. γ 1 and γ 2b refer to human IgG1 and murine IgG2b C region, respectively. Panel B shows ELISA reactivity. Values represent the means of a group of four mice. The antigen used was as follows: γ 1WT (black square); γ 2bWT (open square); and γ 2b V_H^{36-65}

(black circle). Tests were done in duplicate on sera used at 1:800 dilution.

Figure 7 shows PCR amplification and Southern blot hybridization to detect the presence of the transgene H chain *in vivo*. Panel A shows the presence of the transgene in splenic genomic DNA at various times after DNA inoculation. Spleens were harvested 4, 12, 16, 24, 36 and 52 weeks after DNA inoculation. The spleen of a naive mouse served as a negative control and is referred to as time 0. Detection of the transgene in the genomic DNA was performed by PCR amplification using three sets of primers (pCL/pCD, pSE/pNAD and pNEL/pNED) specific for three different DNA fragments of plasmid DNA γ 1NANP and confirmed by Southern blot hybridization using the (³²P)-labeled pNAD oligonucleotide. The location and size of the PCR fragments (VDJ, (NANP)₃ and NESTED) are illustrated in Panel B. A PCR fragment identified as β -actin (for the murine β -actin gene) served as an internal control. Left, the results of PCR amplification. Right, the results of Southern blot hybridization. Panel B is a schematic representation of the V_H gene contained in plasmid DNA γ 1NANP. The annealing sites of the primers, the predicted amplification fragments and their molecular size, are identified: FR, framework region; VDJ refers to a fragment that is inclusive of the coding region for the rearranged VDJ gene segments; (NANP)₃ refers to a 384-bp fragment containing in the CDR3 of the V_H region the sequence coding for three repeats of the tetrapeptide Asn-Ala-Asn-Pro between nucleotides 304 and 340; NESTED refers to a 198-bp fragment inclusive of the coding region for FR3 and the CDR3. +1 refers to the first nucleotide in the coding region of FR1. Any other position in the gene is numbered in reference to nucleotide +1. Panel C shows tissue distribution of the

transgene *in vivo*. Genomic DNA was extracted from the tissues listed. Tissues were obtained at various times from DNA inoculation. Tissues from a naive mouse refers to time 0. Left, PCR amplification of the VDJ fragment of the transgene using the primers of pCL/pDC. Right, results of Southern blot hybridization using the (³²P)-labeled pNAD oligonucleotide.

Figure 8 shows isolation of splenic B and T lymphocytes and detection of the transgene H-chain in the purified lymphocyte populations. B and T lymphocytes from the spleen of DNA-inoculated mice were sorted and purified on a fluorescence-activated cell sorter at the times indicated. Lane 1, fragment amplified with the primers pCL/pCD(VDJ); lane 2, fragment amplified with the primers pSE/pNAD ((NANP)₃); lane 3, fragment amplified with the primers pNEL/pNED (NESTED); lane 4, fragment amplified with the primers pβA1/pβA2(β-actin).

Figure 9 shows the specificity of the primary anti-NANP peptide response after intraspleen inoculation of plasmid DNA. Mice were inoculated with γ1NANP DNA (12 animals) or with control plasmid pSVneo DNA (four animals). Sera were tested by ELISA on synthetic peptide (NANP)_n. Data shown represent the mean of individual titers which were determined on the basis of the last value with an absorbance ≥ of 0.200 (A₄₉₂).

Figure 10 shows the anamnestic response elicited with plasmid γ1NANP DNA following challenge with *P. falciparum* sporozoites. Mice were primed with plasmid DNA γ1NANP or antigenized antibody γ1NANP or antigenized antibody γ1NANP in CFA as indicated. Control groups were inoculated with plasmid γ1WT DNA or saline. On day 45 mice were given a booster immunization with either *P.*

falciparum sporozoites or antigenized antibody γ 1NANP (50 μ g) in IFA subcutaneously as indicated. *P. falciparum* sporozoites were inoculated (10^9) in incomplete DMEM intraperitoneally. Blood samples were collected on day 45 (before the booster injection) and subsequently 15 and 35 days after booster. Antibodies reactive with the synthetic peptide (NANP)_n (panels A and C) and antibodies reactive with the recombinant protein R32LR (panels B and D) were detected by ELISA. Values represent the absorbance (A_{492}) of pooled sera (four mice/group) tested at 1:1600 dilution.

Figure 11 shows challenge with *P. falciparum* sporozoites selectively restimulates clonotypes producing anti-NANP antibodies. Panel A shows the antibody response of mice primed with plasmid γ 1NANP DNA. Panel B shows the antibody response of mice primed with antigenized antibody γ 1NANP. In both instances serum antibodies were detected on microtiter wells coated with synthetic (NANP)_n peptide (black boxes), antigenized antibody γ 1NANP (black triangles), or control synthetic peptide (black circles). Values represent the absorbance (A_{492}) of pooled sera (four mice/group) tested at 1:1600 dilution.

Figure 12 shows isotype analysis of the primary and secondary antibody response against the (NANP)_n peptide in mice primed intrasplenically with plasmid γ 1NANP DNA. Mice received a booster immunization with 105 *P. falciparum* sporozoites (Panel A) or antigenized antibody γ 1NANP (50 μ g) in incomplete Freund's adjuvant (Panel B) on day 45. Columns refer to determinations made in pool of sera from four individual mice. The time of blood collection is indicated. Values represent the

absorbance (A_{492}) of tests done in duplicate on sera used at 1:1600 dilution.

Figure 13 shows a comparison of the primary antibody response in mice following inoculation with plasmid γ 1NANP DNA, or with γ 1NANP protein in immunologic adjuvants. Each group of mice consisted of four animals. Mice given γ 1NANP DNA were inoculated intraspleen. Mice given γ 1NANP protein in alum were injected intraperitoneally. Antibody titers against NANP peptide were measured 6 weeks after inoculation. (Time 0 refers to pre-inoculation bleed.) Data shown represent the mean of individual titers which were determined on the basis of the last value with an absorbance \geq of 0.200 (A_{492}).

Figure 14 shows secondary immune response at various time intervals after priming with plasmid γ 1NANP DNA inoculated intraspleen. All mice were primed intraspleen with plasmid γ 1NANP DNA or control plasmid pSVneo (*) and were boosted *s.c.* by injection of the γ 1NANP protein emulsified in IFA at different times (6-30-104 weeks) after priming. Antibody titers against the NANP peptide were determined 2 and 5 weeks after booster. Among the plasmid γ 1NANP DNA primed mice, ten were boosted after 6 weeks, six were boosted after 30 weeks, and four were boosted after 104 weeks, respectively. Four control mice primed with pSVneo DNA (*) were boosted after 6 weeks. Data shown represent the mean of individual titers which were determined on the basis of the last value with an absorbance \geq of 0.200 (A_{492}).

Figure 15 shows engineering and expression of an immunoglobulin H chain gene with two heterologous epitopes. Panel A shows a schematic representation of the mutagenesis vectors, introduction of the (NANP)₃ and

NANPNVDPNANP coding sequences and partial, nucleotide sequence of CDR2 and CDR3 after insertion. The synthetic oligonucleotides and the mutagenesis steps for the creation of pVH-TAC/CCA are detailed in the Experimental Protocol. Two pairs of complementary synthetic oligonucleotides coding for (NANP)₃ and NANPNVDPNANP, were cloned in the Asp718 site in CDR3 and in the NcoI site in CDR2 of pVH-TAC/CCA. The insertions were verified by dideoxy- chain-termination sequencing. Panel B shows a schematic representation of plasmid DNA $\gamma 1\text{NV}^2\text{NA}^3$ carrying the coding sequences for the two heterologous epitopes in CDR3 and CDR2, respectively. The human $\gamma 1$ constant (C) region gene is in genomic configuration. CH1, CH2, and CH3 refers to the corresponding domains in the C region of the $\gamma 1$ gene. Promoter (Pr) and enhancer (En) elements for tissue-specific expression and the neomycin (Neo^r) and ampicillin (Amp^r) resistance genes are indicated. Panel C shows a schematic representation of antigenized H chain gene product paired with a light chain. The engineered epitopes in CDR3 and CDR2 are as indicated (not to scale).

Figure 16 shows *in vivo* expression of antigenized antibodies $\gamma 1\text{NV}^2\text{NA}^3$. Panel A shows detection of transgene H chain Ig in the serum of mice inoculated with plasmid DNA $\gamma 1\text{NANP}$ (hatched columns) or $\gamma 1\text{NV}^2\text{NA}^3$ (grey columns). Sera were tested at 1:10 dilution. Values (ng/ml) are expressed as the mean (\pm standard deviation) of four mice/group. Panel B shows detection of κ light chain associated with secreted transgene H chain Ig. Results are expressed as absorbance at 492 nm of sera tested at 1:1,600 dilution. Panel C shows detection of λ light chain associated with secreted transgene H chain Ig. Results are expressed as

absorbance at 492 nm of sera tested at 1:1,600 dilution. Time refers to days after DNA inoculation.

Figure 17 shows *in vivo* immunogenicity of CDR3 and CDR2 epitopes. Mice were immunized with plasmid DNA γ 1NANP (black squares) or γ 1NV²NA³ (open squares). Their sera were tested by ELISA on synthetic peptide (NANP)_n (panels A and B) or NANPNVDPNANP (panels C and D). Values refer to absorbance (492 nm) of sera tested at 1:1600 dilution and are expressed as the mean (\pm standard error). Each group consisted of four mice. (*) indicates statistical significance between the values shown in panel B versus panel A. Significance was $p < 0.01$ on day 7, and $p < 0.05$ on day 14. Time refers to days after DNA inoculation.

Figure 18 shows a schematic view of the temporal relationship between detection of the transgene *in vivo*, detection of transgene product and respective antibodies in the circulation, and presence of immunologic memory during STI.

Figure 19 shows a schematic representation of plasmid DNA γ 1NANP, γ 1NANP/GM-CSF and γ 1NANP/IL-2. Panel A shows γ 1NANP is a variant of γ 1WT, the product of the fusion between a human γ 1 C region gene present in the plasmid vector pNeoy1 with murine V_H⁶² gene (2.3 kb) (Sollazzo et al., *supra*, 1989). In this productively rearranged V_H region gene, the CDR3 was modified to code for three repeats of the Asn-Ala-Asn-Pro (NANP) sequence (Sollazzo et al., *supra*, 1990a). The C region gene is in genomic configuration. Panel B shows that, in plasmid DNA γ 1NANP/GM-CSF (granulocyte monocyte-colony stimulating factor), the murine GM-CSF coding sequence was cloned at the 3' end of the CH3 domain. Panel C

shows that, in plasmid DNA γ 1NANP/IL-2, the murine IL-2 coding sequence was similarly cloned at the 3' end of the CH3 domain. Each plasmid DNA carries the regulatory elements, promoter (Pr) and enhancer (En) needed for tissue-specific expression. Neo^r = neomycin and Amp^r = ampicillin, are the resistance genes.

Figure 20 shows the titer of total (Panel A) and IgG1 (Panel B) antibodies against TgIg in mice during STI induced with plasmid DNAs γ 1NANP, γ 1NANP/GM-CSF or γ 1NANP/IL-2 (primary response). Each panel represents an independent experiment. The left panels show values of pooled sera from 6 mice, while center and right panels refer to values of pooled sera from 4 mice each. Antibody titers were determined on the basis of the last dilution with an absorbance (A492) \geq of 0.200. Tests were done in duplicate.

Figure 21 shows isotype class determination of anti-TgIg antibodies in mice inoculated with plasmid DNAs γ 1NANP, γ 1NANP/GM-CSF or γ 1NANP/IL-2. Values refer to a pool of six mice from one out of the three independent experiments shown in Figure (29) 19. Mice were bled at the times indicated. The arrow indicates booster immunization (day 35). The asterisk identifies the elevated IgG1 binding induced by DNA/GM-CSF. GM-CSF augmented the titer of IgG1 antibodies during the secondary response (Log titer 5.0 vs. 4.1). Values are expressed as absorbance (A492). IgM, IgG1 and IgG2b were measured on sera diluted 1:1,600; IgG2a were measured on sera diluted 1:400. Tests were done in duplicate.

Figure 22 shows IgG1 and IgM anti-TgIg antibodies in bone marrow chimeras, *relB* mutants (-/-) and hemizygous (-/+) mice following inoculation with

plasmid DNA/GM-CSF or γ 1NANP DNA. Eight weeks after reconstitution, bone marrow chimeric mice were inoculated with DNA/GM-CSF (Panels A and C) or with DNA (Panels B and D). IgG1 (Panels A and B) and IgM (Panels C and D) antibodies were monitored. Sera were tested at 1:400 dilution. Symbols identify the following groups: bone marrow chimeras (-/+) (black box); bone marrow chimeras (-/-) (open boxes); *relB* hemizygotes (-/+) (black circles); and *relB* mutants (-/-) (open circles). Values shown refer to single mice. Tests were done in duplicate.

Figure 23 shows GM-CSF heightens the anamnestic anti-NANP antibody response following booster immunization with antigenized antibody γ 1NANP. Columns refer to antibody titers (Log 10) were measured on (NANP)n peptide. Experimental groups are identified at the bottom. The arrow indicates the time (day 35) when the booster immunization was given. Values refer to binding of a pool of sera collected at the same time. Each group consisted of four mice.

Figure 24 shows GM-CSF heightens the anamnestic anti-NANP antibody response following booster immunization with *P. falciparum* sporozoites. Columns refer to antibody titers (Log 10) were measured on (NANP)n peptide. Experimental groups are identified at the bottom. The arrow indicates the time (day 45) when the booster immunization was given. Values refer to binding of a pool of sera collected at the same time. Each group consisted of four mice.

Figure 25 shows GM-CSF heightens the anamnestic anti-NANP antibody response following booster immunization with antigenized antibody γ 1NANP in *relB* (-

/-) mice. Antibody titers (Log 10) were measured on (NANP)_n peptide. Mice were primed by inoculation of DNA (open squares) or DNA/GM-CSF (closed squares) as indicated. The arrow indicates the time (day 42) of the booster immunization with γ 1NANP antibody. Values refer to binding of a pool of sera collected at the same time. Each group consisted of four mice. Tests were done in duplicate and repeated at least three times.

Figure 26 shows *relB* (-/-) mice produce IgM antibodies against the whole TgIg following priming with DNA. Antibody titers (Log 10) were measured on γ 1NANP antibody. Mice were primed by inoculation of DNA (open squares) or DNA/GM-CSF (closed squares) as indicated. The arrow indicates the time (day 42) of the booster immunization with γ 1NANP antibody. Values refer to binding of a pool of sera collected at the same time. Each group consisted of four mice. Tests were done in duplicate and repeated at least three times.

Figure 27 shows antigen-specific activation of T lymphocytes by STI. Panel A shows the proliferative response of spleen cells from C57Bl/6 mice inoculated with plasmid DNA g1NANP coding for the B cell epitope (4 mice), γ 1NV²NA³ coding for the B and T cell epitopes (4 mice), or control plasmid pSV2neo (2 mice), and harvested on day 7. Cells were cultured in the presence of the antigens indicated along the abscissa. Results refer to stimulation index expressed as the mean \pm S.D.. Results correspond to two independent experiments. AgAb = antigenized antibody. Tests were run in triplicate. Panel B shows IL-2 production in spleen cell cultures from the same C57Bl/6 mice shown in panel A. Results are expressed as counts per minute (cpm) of the proliferative

response of indicator NK.3 cells and are expressed as the mean \pm S.D..

Figure 28 shows kinetics of T cell activation *in vivo*. The proliferative response of spleen cells from C57Bl/6 mice inoculated with plasmid DNA was determined. Spleen cells were harvested on day 3, 7, 14 and 21. Cells were cultured in the presence of the synthetic peptide corresponding to the Th cell determinant (upper panel) or the B cell epitope (lower panel), as a control. Results refer to stimulation index. At each time point, groups consisted of 2 mice for the DNA coding for heterologous epitopes and one inoculated with plasmid pSV2neo control.

Figure 29 shows levels of IFN- γ and IL-4 during the primary response. Spleen cells harvested 7 and 14 days after immunization were incubated with synthetic peptide corresponding to the Th cell determinant (50 μ g/ml) for 40 hours. Supernatants from triplicate cultures were harvested and tested in capture ELISA specific for IFN- γ or IL-4.

Figure 30 shows activated cells are CD4⁺ T cells. Seven days after DNA inoculation, spleen cell populations were prepared and depleted of CD8⁺ (Panel C) or CD4⁺ (Panel D) cells by antibody plus complement. Unseparated CD8⁺ cells (Panel A) and unseparated CD4⁺ cells (Panel B) are shown as reference. The proliferative response (Panel E) and IL-2 production (Panel F) of unfractionated (total), separated CD4 and CD8, and reconstituted (CD4+CD8) T cell populations are shown. Stimulation indexes and IL-2 production were determined.

Figure 31 shows T cell immunity induced by intraspleen DNA inoculation spreads to lymph nodes. Cell proliferation (Panel A) and IL-2 production (Panel B) in a pool of inguinal, mesenteric and cervical lymph node, and spleen cells harvested 7, 14 or 21 days after γ 1NV²NA³ DNA inoculation. Lymph nodes were isolated from four mice/experiment. Serum transgenic Ig (ng/ml) in the serum is expressed as the mean \pm SD of six different mice at each time point (Panel C). Cell proliferation (Panel D) and IL-2 production (Panel E) of lymph nodes collected from (1) axillary, brachial, deep and superficial cervical (upper); (2) mesenteric, renal and epigastric (middle): and (3) popliteal, caudal, sciatic and lumbar (lower), lymph nodes 14 days after DNA inoculation. Lymph nodes were isolated from six mice.

Figure 32 shows the effect of linked recognition of Th and B cell epitopes on the antibody response. Titer (Log) of B-cell epitope reactive antibodies in mice inoculated with plasmid DNA coding for T and B epitopes (triangle), B cell epitope (square) or control plasmid (circle) (Panel A). The titer (Log) of IgG1, IgM and IgG2a antibodies determined in ELISA in the sera of mice inoculated with plasmid DNA coding for the B-cell epitope only (Panel B) or with plasmid DNA coding for the B- and T cell epitopes (Panel C). Every symbol refer to a single mouse. All mice were tested on day 14. Tests were done in duplicate.

Figure 33 shows a schematic representation of plasmid DNA γ 1NP. This H-chain coding plasmid is the product of the fusion of a human γ 1C region with a murine V_H engineered to express the 13 amino acid residues from the sequence of the influenza virus nucleoprotein (NP) antigen (366-379) in the third complementarity-

determining region (CDR3). This NP peptide is presented in association with the D^b allele in H-2^b mice. The coding strand of the CDR3 region is shown in bold, with the NP-coding sequence underlined. The amino acid sequence of the influenza peptide ³⁶⁶ASNENMETMESSTL³⁷⁹ is shown in bold. B, BamHI; RI, EcoRI; Neo, neomycin (G418) resistance; Amp, ampicillin resistance. The H-chain gene was mutagenized to introduce a single KpnI/Asp718 site and complementary oligonucleotides 5' GTA CCC GCT TCC AAT GAA AAT ATG GAG ACT ATG GAA TCA AGT ACA CTT 3', 5' GTA CAA GTG TAC TTG ATT CCA TAG TCT CCA TAT TTT CAT TGG AAG CGG 3' coding for residues 366-379 of the influenza virus NP antigen (ASNENMETMESSTL) were introduced between 94V and 95P of the mutagenized V_H region. The engineered V_HNP coded by the 2.3 kb EcoRI fragments was cloned upstream from a human γ 1 constant (C) region gene contained in the 12.8 kb vector pN γ 1

Figure 34 shows survival curves in mice vaccinated with plasmid DNA γ 1NP (DNA) via intraspleen inoculation and challenged with x10LD₅₀ influenza virus. Other groups were primed with plasmid DNA γ 1NP followed by a booster with synthetic peptide the influenza virus NP antigen ASNENMETMESSTL in immunologic adjuvant (DNA + peptide), or NP synthetic peptide ASNENMETMESSTL in immunologic adjuvant followed by a booster with the same synthetic peptide (peptide + peptide). Challenge with the virus was given three months after priming.

Figure 35 exemplifies the engineering of an immunoglobulin H chain gene with two heterologous Th cell epitopes. The H chain gene is coded by plasmid vector γ 1NV2VTSA3. The V_H region is the 2.3 kb Eco RI genomic

fragment containing the VDJ rearrangement of a murine V region gene (see Figure 1 for detail). The human $\gamma 1$ constant (C) region gene is in genomic configuration. CH1, CH2, and CH3 refers to the corresponding domains in the C region of the $\gamma 1$ gene. Promoter (Pr) and enhancer (En) elements for tissue-specific expression and the neomycin (Neo^r) and ampicillin (Amp^r) resistance genes are indicated. The V_H region is modified by mutagenesis to code for two heterologous determinants as indicated in the right panel. The arrow points the structure of the translated protein composed of the transgenic H chain and a light (L) chain provided by the host cell. The amino acid sequences in the CDR2 and CDR3, are indicated and correspond to the Th cell determinant NANPNVDPNANP from the outer coat of the malaria parasite *P. falciparum* (in CDR2) and the VTSAPDTRPAP epitope from the tandem repeat of the tumor antigen MUC-1 (in CDR3). CDR= complementarity determining region. H = heavy (chain); C = constant region. Not to scale.

Figure 36 shows the effect of linked recognition of a dominant Th epitope and a cryptic/subdominant Th epitope on the proliferative response to the cryptic/subdominant epitope. Th/Th associative recognition is necessary to render immunogenic T cell determinant from the MUC-1 antigen. Mice were inoculated with plasmid DNA as indicated. Spleen cells were harvested on day 15 and re-stimulated *in vitro* for 4 days in the presence of 50 μ g/ml of synthetic peptide (DTRP)3 and VTSAPDTRPAP (denoted as VTSA). Both sequences are contained in the PDTRPAPGSTAP tandem repeat of the tumor antigen MUC-1. Superscript numbers indicate the CDR in which the heterologous antigen sequence has been inserted. Subscript numbers indicate the number of times the sequence in brackets is

repeated in the context of a particular CDR. The results shown are cumulative of three independent experiments. Each group is constituted of 8-10 mice. Results are expressed as stimulation index. Bars indicate means of stimulation indexes \pm SEM.

Figure 37 exemplifies spontaneous somatic transgenesis in human B cells. A) Raji (MHC class II⁺) and RJ2.2.5 (a MHC class II⁻ variant) cells were plated at various concentrations in 300 μ l of phosphate buffered saline with 5 μ g of plasmid DNA (γ 1NANP) and incubated at 37°C for 1 hour in a 5% CO₂ atmosphere. Next the cells were washed and cultured in complete medium at 37°C for 24 hours. Genomic DNA was extracted using the QIAamp Blood Kit (Qiagen) and subject to a two-round nested PCR using VDJ specific primers. The PCR products were analyzed on a 1% agarose gel with ethidium bromide stain. After 24 hours the transgene was detected by PCR in both the Raji and RJ2.2.5 cells (panel A). PCR products of genomic DNA extracted from transfected Raji and RJ2.2.5 cells. Lane 1, 10⁵ Raji cells transfected with 5 μ g DNA; lane 2, 10⁴ Raji cells; lane 3, 10³ Raji cells; lane 4, 10² Raji cells; lane 5, 10⁵ RJ2.2.5 cells; lane 6, 10⁴ RJ2.2.5 cells; lane 7, 10³ RJ2.2.5 cells; lane 8, 10² RJ2.2.5 cells; lane 9, 10⁵ untransfected Raji cells; lane 10, H₂O; lane 11, γ 1NANP plasmid; lane 12, 1 kb ladder. B) RT-PCR products of transfected Raji cells (10⁵) following total RNA isolation. Lane 1, transfected Raji; lane 2, transfectoma cells; lane 3, untransfected Raji; lane 4, transfected RJ2.2.5; lane 5, plasmid DNA (positive control for PCR); lane 6, H₂O; lane 7, 1 kb ladder. A murine transfectoma cell line was used as a (+) control. B) The occurrence of transcription is also documented (panel B). The total RNA of 10⁵ transfected cells was extracted in a single-step after 7 day culture using

guanidinium thiocyanate phenol-chloroform. RNA coding for the H chain transgene product was detected by RT-PCR in transfected Raji cells.

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DETAILED DESCRIPTION OF THE INVENTION

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The present invention provides a rational and effective approach to immunization and is predicated on the induction of antibody and T cell responses following inoculation of a polypeptide encoded by a nucleic acid molecule, for example, an immunoglobulin H chain gene, targeted to hematopoietic cells such as lymphocytes in a lymphoid tissue. The methods of the invention can be used to initiate immunity, establish immunologic memory and program the immune response in a reproducible way from a single inoculation of a nucleic acid molecule such as plasmid DNA.

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The invention provides a method for stimulating an immune response, comprising administering to a lymphoid tissue a nucleic acid molecule comprising an expression element operationally linked to a nucleic acid sequence encoding one or more heterologous epitopes.

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The methods of the invention are based on an effective method for delivering a nucleic acid molecule, which can serve as a vaccine, to primarily but not exclusively B cells, which can preferably be in secondary lymphoid organs, and hence program the immune system to produce large amounts of immunogenic molecules. The method for delivering a nucleic acid molecule such as a DNA vaccine is termed somatic transgene immunization (STI). The methods of the invention are effective at stimulating an immune response because the nucleic acid molecule is targeted to hematopoietic cells such as B

lymphocytes in lymphoid tissues. The effectiveness of the methods result from the self-renewing property of antigenized antibody genes harbored in B lymphocytes and the constitutive ability of activated B lymphocytes to synthesize many copies of transgene products. STI can be used as a vaccination process that is designed to exploit one of the body's most efficient and exquisite machineries for protein production.

In one embodiment, the variable region of antibodies can be re-engineered to code for discrete sequences of heterologous antigens to impart the molecule new antigenic and immunogenic properties, called antibody antigenization. This approach allows modification of the complementarity determining regions (CDR) of the variable domain of an immunoglobulin so that, after antigenization, antibodies become structural mimics of antigens in a way that leads to induction of B-cell and T-cell immunity. Consequently, inoculation of antigenized H chain genes and synthesis of transgenic Ig by the host during STI is a way to provide the organism with exogenous B-cell and T-cell epitopes. Methods of generating antigenized immunoglobulins is described, for example, in U.S. patents 5,583,202, issued December 10, 1996, and 5,658,762, issued August 19, 1997.

The present invention provides the combined use of STI and antigenized antibody genes as a new method to induce antigen-specific immunity, antibody and T cell mediated. In addition to antigenized antibodies, the methods of the invention for stimulating an immune response can use a nucleic acid molecule expressing one or more heterologous polypeptides. The heterologous polypeptide is operationally linked to an expression element allowing expression of the polypeptide in all

targets in a lymphoid tissue. Similar to an antigenized antibody, the methods exploit the polypeptide expression capabilities of hematopoietic cells targeted upon administration of a nucleic acid molecule to a lymphoid tissue. The heterologous polypeptide can encode one or more epitopes capable of eliciting an immune response.

The methods of the invention are useful, for example, for stimulating an immune response against pathogens, tumor antigens and pathological processes. The present invention can be used to stimulate an immune response against infectious agents including, viruses, for example, immunodeficiency virus 1 and 2, hepatitis viruses, papilloma virus, influenza virus, Epstein-Barr virus, cytomegalovirus, Japanese encephalitis virus, Dengue virus, and other retroviruses/lentiviruses; protozoa, for example, parasites causing malaria, leishmaniasis, trypanosomiasis, filariasis, toxoplasmosis, hookworm, tapeworm; yeast, for example, *Candida albicans*; bacteria, in particular pathogenic bacteria such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and bacteria that cause colera, *Mycoplasma/Ureaplasma*, and spirochetes such as treponema pallidum, borrelia, leptospira; toxins, for example, botulinum, anthrax, snake toxins, insect toxins, and warfare-related chemical toxins.

The methods of the invention can also be used to stimulate an immune response to pathological or disease conditions. The pathological or disease conditions can be, for example, tumors, including those expressing antigens such as prostate specific antigen (PSA), Her-2/neu, p53, MUC-1, telomerase, carcinoembryonic antigen (CEA), melanoma associated antigens (MAGE), thyrosinase, gp100; autoimmune diseases,

for example, diabetes, myasthenia gravis, multiple sclerosis, rheumatoid arthritis, Crohn's disease, uveitis; allergy, for example, dermatitis and asthma; metabolic disorders, for example, hypertension, diabetes, hypercholesterolemia; endocrine disorders, for example of the thyroid, adrenals, pituitary, ovary, testis; mental disorders, for example, bipolar disorders, schizophrenia; pain, for example, modulation of neurotransmitters and neuropeptides; blood disorders, for example, coagulation, anemias, thrombocytopenia; and dental disorders, for example, caries. The methods of the invention can also be used to control reproduction, for example, contraceptive vaccination. The methods of the invention can additionally be used for treating transplant patients, for example, solid organ by inducing transplantation, and bone marrow transplantation, anti-HLA immunity. The present invention can be used for the production of human and animal vaccines against viruses, parasites, bacteria, allergy, autoimmune disease, and tumors. The methods of the invention are useful for stimulating an immune response to treat or prevent a condition as described above.

The methods of the invention include the step of administering a nucleic acid molecule encoding one or more heterologous epitopes to a secondary lymphoid tissue. The secondary lymphoid tissue can be spleen, lymph nodes, mucosa-associated lymphoid tissue (MALT), including tonsils and Payer's patches, and the nasal-associated lymphoid tissue (NALT) such as the Waldeyer's ring, and the urogenital lymphoid tissue. A variety of methods can be used to administer a nucleic acid molecule to a lymphoid tissue. For example, a nucleic acid molecule can be directly injected into a lymphoid tissue such as a lymph node. A nucleic acid molecule can also

be directly injected into the spleen of an individual, for example, using endoscopy-guided fine needle injection. Additional methods include the intravenous injection of DNA encapsulated into (immuno)-liposomes or biodegradable beads of various chemical structure for time-controlled release, for example, hyaluronic acid. Additional methods include the (intra)-nasal delivery of DNA encapsulated into (immuno)-liposomes or biodegradable beads or various chemical structure for time-controlled release such as hyaluronic acid. Additional methods include the oral delivery of DNA encapsulated into (immuno)-liposomes or biodegradable beads or various chemical structure for time-controlled release, for example, hyaluronic acid, in a suitable acid-resistant pharmaceutical vehicle, or engineered in live attenuated bacteria, for example, *Salmonella typhi*.

As used herein, the term "epitope" refers to a molecule or fragment thereof capable of stimulating an immune response. A polypeptide epitope is at least three amino acids in length for antibody responses and at least eight amino acids in length for T cell responses.

As used herein, the term "heterologous polypeptide" when used in reference to a nucleic acid molecule means that the polypeptide is encoded by a nucleic acid sequence operationally linked to an expression element, where the polypeptide is not naturally found linked to the expression element. As such, the polypeptide is heterologous to the expression element.

Similarly, the term "heterologous epitope" refers to an epitope encoded by a nucleic acid sequence operationally linked to an expression element, where the

epitope is not naturally found linked to the expression element. When a heterologous epitope is contained in an immunoglobulin, the epitope is not ordinarily found in the immunoglobulin. Hence, the immunoglobulin contains a heterologous epitope sequence. Such heterologous epitope sequences can include antigenic epitopes as well as receptor-like binding domains or binding regions that function as receptor sites, for example, the human CD4 binding domain for HIV, hormone receptor binding ligands, retinoid receptor binding ligands and ligands or receptors that mediate cell adhesion.

The epitope encoded by the nucleic acid molecules of the invention is operationally linked to an expression element. As used herein, an "expression element" is a nucleic acid regulatory element capable of directing expression of genetic element such as an epitope. An expression element can include, for example, promoters and/or enhancers capable of allowing expression of an operationally linked genetic element such as a genetic element encoding a polypeptide or epitope. Particularly useful promoters and enhancers are those that function in hematopoietic cells, termed "hematopoietic cell expression elements." Such hematopoietic expression elements are capable of allowing expression in a cell of hematopoietic origin, for example, a B cell, T cell or dendritic cell. These promoters and enhancers can be specific for a hematopoietic cell, or they can function in hematopoietic cells as well as other cell types.

The methods of the invention can employ a nucleic acid molecule encoding an epitope expressed as a fusion with a cytokine. The cytokine can be any cytokine, including, for example, granulocyte-macrophage

colony-stimulating factor (GM-CSF), interleukin-2 (IL-2), interleukin-4 (IL-4), interferon- γ (INF- γ), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-12 (IL-12) and the like. Alternatively, a nucleic acid molecule such as plasmid DNA coding for an antigenized H chain gene can be concomitantly administered with a nucleic acid molecule such as plasmid DNA coding for a cytokine. One skilled in the art can select a cytokine for fusion with a polypeptide based on a desired type of immune response which can depend on the type of antigen used to elicit an immune response.

The nucleic acid molecule used in the invention can encode an immunoglobulin molecule containing one or more heterologous epitopes. The epitopes can be inserted into a complementarity-determining region (CDR) of the immunoglobulin molecule (see, for example, Kabat et al., Proteins of Immunological Interest, U.S. Department of Health and Human Services, Bethesda MD (1987)). The epitope can be inserted within CDR1, CDR2 and/or CDR3. Furthermore, one or more epitopes can be inserted within any of the CDRs. Thus, the same epitope can be inserted multiple times within a single CDR or can be inserted multiple times within different CDRs. Different epitopes can also be inserted within the same CDR or can be inserted within different CDRs. Thus, a single CDR can have a single epitope, multiple copies of the same epitope, or two or more different epitopes in the same CDR. It is likely that as many as 6 epitopes, or possibly more, can be inserted into the three CDRs of a variable region. These methods utilize antigenized immunoglobulins which are described in U.S. patents 5,583,202 and 5,658,762.

Generally, when more than one epitope is administered to stimulate an immune response, the multiple epitopes are encoded on the same nucleic acid molecule. When encoded on the same plasmid, the multiple epitopes can be operationally linked to the same expression element and expressed as a fusion polypeptide, or the multiple epitopes can be expressed from multiple copies of the expression element. Multiple epitopes can also be expressed from different expression elements.

Furthermore, the same epitope can be administered in different nucleic acid molecules such as different plasmids. Similarly, different epitopes can be administered in one nucleic acid molecule or can be administered in multiple nucleic acid molecules such as on different plasmids. Using different nucleic acid molecules encoding multiple epitopes allows the administration of many more epitopes than could be encoded on a single nucleic acid molecule.

The immunoglobulin molecules useful in the invention can contain the variable region of a heavy or light chain, or a functional fragment thereof. For example, a single CDR can be a functional fragment if the immunoglobulin, as used herein as an antigenized antibody, functions to stimulate an immune response. The immunoglobulin can also comprise two or three CDRs or a variable region as described above. Additionally, the immunoglobulin molecules useful in the invention can be a heavy chain or a light chain. The effector function of the immunoglobulin molecule can be conferred by the constant region of the immunoglobulin molecule. Therefore, the immunoglobulin molecule can include a constant region. The constant region can be derived, for example, from human, primate, mouse, rat, chicken or camel, as desired. However, it is understood that a

constant region is not required for the immunoglobulin of the invention if the functional fragment of the immunoglobulin functions to stimulate an immune response.

5 The invention also provides a nucleic acid molecule comprising an expression element operationally linked to a nucleic acid sequence encoding one or more heterologous polypeptides. The heterologous polypeptide can function as one or more epitopes. Furthermore, the
10 epitope can be expressed as a fusion with a cytokine. When an epitope is expressed as a fusion polypeptide, for example, a fusion with a cytokine, the epitope can be fused proximal to a cytokine, or there can be intervening sequence between the epitope and the cytokine. The
15 cytokine can be, for example, GM-CSF, IL-2, IL-4, INF- γ , IL-5, IL-6, IL-10 and IL-12. The expression element of the nucleic acid molecules of the invention can be a hematopoietic expression element.

20 The methods of the invention can be used to stimulate an immune response. The immune response elicited can be an antibody response, a CD4 T cell response or a CD8 T cell response. Two major classes of T cells, termed T helper cells and T cytotoxic cells, can
25 be distinguished. The classification of T cells into T helper cells and T cytotoxic cells is generally based on the presence of either CD4 or CD8 protein, respectively, on the cell surface. The methods of the invention can be used to elicit an antibody response, a CD4 T cell
30 response or a CD8 T cell response, or any combination of two or more of these responses, including all three responses. For example, the methods of the invention can be used to stimulate an antibody response and a CD4 T
35 cell response. The methods of the invention can also be used to stimulate an antibody response and a CD8 T cell

response. Additionally, the methods of the invention can be used to stimulate a CD4 T cell response and a CD8 T cell response. Furthermore, the methods of the invention can be used to stimulate an antibody response, a CD4 T cell response and a CD8 T cell response. In addition, the methods of the invention can be used to stimulate multiple CD4 T cell responses, for example, two or more, three or more, or five or more CD4 T cell responses. Similarly, multiple CD8 T cell responses can be stimulated using methods of the invention. Thus, depending on the type of immune response desired for a given type of antigen or condition, one skilled in the art can select the most appropriate immune response, an antibody, CD4 T cell or CD8 T cell response, to provide an optimized immune response for a given condition or potential condition.

The success of DNA vaccination is determined by a series of factors, all of which depend on the efficiency of gene delivery and gene expression *in vivo*. Strategies have been developed for receptor-mediated gene delivery (Wu et al., J. Biol. Chem. 264:16985-16987 (1989)) to exploit specific structures on somatic cells and their mechanisms to internalize macromolecules. Moreover, targeted delivery and expression of DNA can also be gauged through the specificity of regulatory elements such as promoters and enhancers, which allow the transgene to be transcribed and translated in tissues.

The methods disclosed herein were developed keeping in mind factors such as the efficiency of *in vivo* transfection, including DNA uptake by host cells, the efficiency with which transfected cells utilize the DNA and synthesize the transgene product, and the ability of *in vivo* transfected cells to serve as antigen-presenting

cells. Since during natural infection pathogens activate lymphocytes in lymphoid organs, likewise it was likely that the most effective way to mimic natural infection would be to direct immunization to sites of immune-
5 response induction using transgenes under the control of lymphoid tissue-specific regulatory elements.

As disclosed herein, the inoculation of plasmid DNA coding for a rearranged immunoglobulin (Ig) H chain
10 gene with B cell-specific promoter and enhancer elements can be used to stimulate an immune response, leads to expression of these genes in B lymphocytes. Specifically, this approach reaches two objectives: exploit B lymphocytes as powerful minifactories of
15 antigenic material and use them as antigen-presenting cells (APC). Therefore, efficient utilization of the foreign DNA and antigen presentation by the very cells harboring the transgene is addressed in one operational event. Thus, the targeting of nucleic acid molecules
20 encoding a heterologous epitope to a lymphoid tissue exploits the natural high level expression of immunoglobulins in B lymphocytes.

As disclosed herein, a single inoculation of
25 the H chain gene targeted to spleen lymphocytes is sufficient to initiate immunity (see Example I), establish immunologic memory (see Example V), and program the immune response predictably and reproducibly (see Examples II and IV). Experiments in murine systems, *in*
30 *vitro* and *in vivo*, demonstrate that the H chain polypeptides of the transgene associate with endogenous light chains (Example IV), and transgenic Ig are invariably secreted in amounts between 15 and 30 ng/ml (Example I). The synthesis of transgenic Ig is followed
35 by an immune response consisting in antibodies and T

cells specific for antigenic determinants of transgenic Ig by day 5-7. The antibody response remains detectable almost indefinitely. Upon booster injection with an appropriate antigen, a typical secondary immune response is induced. This process is termed somatic transgene immunization (STI) (see Example I).

In its simplest form STI is reflected by a model in which plasmid DNA is injected directly into a lymphoid organ where it reaches follicles and within them, the B lymphocytes. The hypothetical scenario that follows is described hereunder. A first set of B lymphocytes (B1) uptake and immediately begin utilizing the H chain DNA molecule which undergoes transcription and translation into a functional H chain. A fraction of nascent H chain polypeptides is secreted in association with endogenous L chain as transgenic Ig carrying heterologous epitopes (antigenized transgenic Ig). This event sets in motion the immune response. A second set of B lymphocytes (B2) respond to the antigenic determinants of secreted transgenic Ig by producing antibodies. Transgenic Ig also act by activating Th cells. T cell determinant peptides are processed and presented either by B lymphocytes harboring the transgene (direct presentation) or by proximal interdigitating dendritic cells (IDC) (cross-priming). The process of immunity spreads rapidly to other secondary lymphoid organs through secreted transgenic Ig reaching the bloodstream and the lymphatic system. Activated T cells can follow a similar pattern and emigrate from the initial site of activation to encounter the same peptides carried in the form of transgenic Ig in distal lymphoid organs. As the response evolves in time, transgenic Ig alone or complexed with specific antibodies are trapped

by follicular dendritic cells (FDC) and stored along the dendrites to be re-utilized during memory responses.

B lymphocytes are the target cell population of STI as demonstrated by PCR-amplification of genomic DNA from purified B cells (see Example II). Although the mechanism of DNA uptake by B cells is not completely understood, it is likely that internalization is mediated either by membrane Ig with anti-DNA reactivity, which exist in the normal B cell repertoire (Holmberg et al., Immunol. Rev. 93:147-169 (1986); Glotz et al., J. Immunol. 141:383-390 (1988)), by a non-Ig receptor for DNA (Bennett et al., J. Clin. Invest. 76:2182-2190 (1985)), or by the class II HLA molecule (Filaci et al., Eur. J. Immunol., 28:3968-3979, (1999)).

The variable region of antibodies can be re-engineered to code for discrete sequences of heterologous antigens to impart the molecule new antigenic and immunogenic properties, antibody antigenization (Zanetti, Nature 355:466-477 (1992)). This approach enables one to modify *ad hoc* the complementarity-determining regions of the variable (V) domain so that, following antigenization, antibodies become structural mimics of antigens in a way that leads to induction of B-cell (Billette et al., Proc. Natl. Acad. Sci. USA 88:4713-4717 (1991); Lanza et al., Proc. Natl. Acad. Sci. USA 90:11683-11687 (1993); Rigaudy et al., DNA Cell Biol. 13:585-591 (1994); Zaghoulani et al., Proc. Natl. Acad. Sci. USA 92:631-635 (1995); Cook and Barber, Vaccine 13:1770-1778 (1995); Cortes et al., J. Biol. Chem. 271:33670-33677 (1996); Cook and Barber, Aids Res. Hum. Retroviruses 13:449-460 (1997)) and T-cell (see Example IV and Zanetti et al., Immunol. Rev. 130:125-150 (1992); Zaghoulani et al., J. Immunol. 148:3604-3609 (1992);

Zaghouani et al., Science 259:224-227 (1993); Billetta et al., Eur. J. Immunol. 25:776-783 (1995) immunity.

Consequently, inoculation of antigenized H chain genes and synthesis of transgenic Ig by the host during STI is a way to provide the organism with exogenous B- and T-cell epitopes.

In transgenic Ig, B-cell epitopes are expressed with controlled geometry and spatial characteristics to approximate the shape of native antigens from which they derive. Since the antigen receptor on B lymphocytes recognizes antigens through their three-dimensional structure and binds establishing interactions over large sterically and electrostatically complementary areas, the expression of B cell epitopes in antibody loops induce antibodies cross-reactive with a native structure. It has been demonstrated that heterologous peptides conformationally-constrained within antibody CDR loops have sufficient structure stability and conformation to interact with somatic receptors in a discriminating way (Billetta et al., *supra*, 1991; Lanza et al., *supra*, 1993; Rigaudy et al., *supra*, 1994; Zaghouani et al., *supra*, 1995; Cook et al., *supra*, 1995; Corthesy et al., *supra*, 1996; Cook et al., *supra*, 1997). Activation of T cells, on the other hand, requires that antigen be presented in the form of small peptides. As disclosed herein, T-cell peptides expressed in CDR loops are easily processed and presented in the context of major histo-compatibility complex (MHC) molecules (see Example IV and Zanetti et al., *supra*, 1992; Zaghouani et al., *supra*, 1992; Zaghouani et al., *supra*, 1993; Billetta et al., *supra*, 1995). Thus, during somatic transgenesis, B cells harboring and synthesizing transgenic Ig become a self-renewing source of T cell peptides. Processing and

presentation is mediated by B cells directly or through cross-priming of other antigen presenting cells (APC).

The dual immunogenic potential of transgenic Ig is best understood by analyzing the intracellular journey of nascent antigenized H chain polypeptides. These polypeptides are subject to quality control mechanisms within the endoplasmic reticulum (Sitia and Cattaneo, Synthesis and assembly of antibodies in natural and artificial environments, Hardwood Academic Publishers, Luxembourg (1995)). Newly formed H chain polypeptides that are properly folded and associate with the endogenous light chain they proceed through secretion along the secretory pathway. The secreted molecules have direct bearing on the induction of B cell immunity. Alternatively, misfolded H chains are subject to degradation by local proteases (Klausner and Sitia, Cell 62:611-614 (1990)), giving origin to peptides, including those in the 9-15 amino acid range, which combine with MHC molecules and are exported to the cell surface. Cumulative data suggest that CDR loops may be sites for preferential proteolytic cleavage within the V region. Neither the position of heterologous peptides relative to the N terminus nor the flanking residues appear to influence this process and overall presentation to T lymphocytes.

When a transgene coding for a strong Th cell determinant is inoculated into mice, a vigorous CD4 T cell response is elicited (Gerloni et al., J. Immunol., 162:3782-3789 (1999)). The activation of Th cells is reproducible and always hallmarked by the concomitant production of large amounts of IL-2 and proportional amounts of IFN- γ and IL-4. Conventional DNA immunization favors Th1 responses (Roman et al., *supra*, 1997; Chu et

al., J. Exp. Med. 186:1623-1631 (1997)). Thus, STI activates uncommitted CD4 T cells.

As to class I MHC presentation, earlier studies had shown that B lymphoma cells engineered with a H chain transgene coding for a virus CTL epitope present the class I peptide to a T cell clone specifically (Zanetti et al., *supra*, 1992; Billetta et al., *supra*, 1995). In mice inoculated with the same transgene, a specific CD8 T response was measured. The results disclosed herein indicate that STI serves as an endogenous source of T cell peptides and has fulfilled basic requirements for immunogenicity *in vivo* (see Example IX).

Collectively, the results disclosed herein indicate that STI is an effective system for inciting immunity *in vivo*. STI induces immunity using B cells for the protracted manufacturing of immunogenic molecules (a B cell can produce 10^3 molecules of antibody/second (Langman and Cohn, Mol. Immunol. 24:675-697 (1987)), without harming the immune system in the process. Moreover, STI can be used to direct the synthesis of heterologous antigenic determinants by B lymphocytes as a self-determined event. STI integrates the immune system itself into the immunization process: B lymphocytes produce immunogenic molecules of predetermined antigenicity and function as APC.

Secreted transgenic Ig can target APC via the Fc receptor for secondary antigen processing and presentation, hence acting as source of antigen peptides for lymphoid tissues distal from the site of initiation of immunity. From this it is easy to see how immunity can spread from the initial site. In fact, cells harboring the transgene do not colonize other lymphoid

5 Unlike conventional immunization systems, where antigen or antigen peptide in immunologic adjuvant activate T cells only in draining lymph nodes (Kearney et al., Immunity 1:327-339(1994), during STI, mobilization of activated T cells together with the diffusion of soluble
10 transgenic Ig facilitate spreading of T cell immunity throughout the body (see Example VIII).

15 involving only a discrete number of B cells (fewer than
0.07 %). The results disclosed herein indicate that the
physiological machinery provided by STI can be exploited
to program immune response in an individual. STI has been
exploited to identify cell types and dissect cellular
20 networks during the immune response *in vivo* without
interference by immunologic adjuvants. For instance, the
IgM to IgG1 switch can be directed *in vivo* by inoculating
a transgene chimerized with GM-CSF (Example VI) or by
including a strong Th cell determinant (Zanetti et al.,
25 *supra*, 1998). The role of STI in the establishment of
immunological memory has also been characterized (Example
VII).

30 of the transgene, synthesis of the corresponding gene
product, its presentation to immunocompetent cells and
induction of immunity occur after repeated inoculations
of plasmid DNA (Whalen and Davis, Clin. Immunol.
Immunopathol. 75:1-12 (1995)). Most previous studies
35 have targeted the muscle, a tissue with low replicative

activity, using genes under the control of foreign viral promoters to initiate their transcription. In selected studies, the transgene has been shown to persist in the muscle in a non-integrated, extrachromosomal form for a long time (Nichols et al., *Ann. N. Y. Acad. Sci.* 772:30-39 (1995); Wolff et al., *Hum. Mol. Genet.* 1:363-369(1992)), albeit the most efficient gene expression is achieved during active muscle generation (Wells and Goldspink, *Febs Lett.* 306:203-205 (1992)) or regeneration (Davis et al., *Ann. N. Y. Acad. Sci.* 772:21-29 (1995); Davis et al., *Hum. Gene Ther.* 4:151-159 (1993)). Targeting of plasmid DNA using tissue-specific regulatory elements for transcription has been done in a few instances only (Perales et al., *Proc. Natl. Acad. Sci. USA* 91:4086-4090(1994)).

During immunity to natural infection, lymphocytes are in general activated by the pathogen or by APCs in lymphoid organs. Mimicking natural infection through inoculation of genes under the control of tissue-specific regulatory elements directly targeted to a lymphoid tissue can help elucidate the way nucleic acids initiate immunity.

As disclosed herein, DNA inoculation was tested by directly inoculating into a lymphoid organ, the spleen. The plasmid DNA coding for an immunoglobulin heavy (H) chain gene under the control of tissue-specific promoter and enhancer elements was used (Banerji et al., *Cell* 33:729-740 (1983); Gillies et al., *Cell* 33:717-728 (1983); Grosschedl and Baltimore, *Cell* 41:885-897 (1985); Mason et al., *Cell* 41:479-487 (1985)). B lymphocytes, which are able to utilize the transgene physiologically and serve as APCs (Lanzavecchia, *Nature* 314:537-539 (1985)), are the cell type targeted in STI.

The methods disclosed herein can be used for immunization and can be used to study the immunogenicity of immunoglobulins in a way that is independent of the physical state of the protein and of the use of immunological adjuvants.

Immunization by inoculation of somatic cells with nucleic acids offers the possibility to evoke specific and often strong immune responses in the absence of immunological adjuvants (see Example I; Cohen, Science 259:1691-1692 (1993)). As disclosed herein, immunization with DNA should be not only possible but likely more effective using a gene possessing tissue-specific regulatory elements directly targeted to cells that can function as antigen-secreting as well as antigen-presenting cells (see Example I and VIII).

As disclosed herein, inoculation of somatic cells with nucleic acids results in tissue-specific targeting. Using STI successful initiation of immunity was achieved with *in vivo* targeting of a tissue with high replicative activity using a gene under the control of tissue-specific promoter and enhancer elements (Example I). The results presented herein are in contrast to what was previously known about DNA immunization, where DNA under the control of viral promoters inoculated into tissues with low replicative activity, for example, the muscle, from which the transgene or its product need to travel to the draining lymph node(s) in order to initiate an adaptive immune response (Nichols et al., *supra*, 1995; Wolff et al., *supra*, 1992).

As disclosed herein, direct targeting of the spleen with a gene under the control of lymphoid tissue-specific promoter and enhancer elements led to detection

of transgene mRNA for at least four months, a much longer time compared with the few days reported for transfection of DNA under the control of a viral promoter into actively replicating cells (Sikes et al., Hum. Gene Ther. 5:837-844 (1994)).

Hypermutation in the VDJ region of the H chain transgene. Hypermutation usually occurs as the result of affinity maturation of antibodies during the course of the immune response and is localized around the V segment (Griffiths et al., *supra*, 1984). Nucleotide sequence analysis of VDJ coding regions present in genomic DNA of inoculated spleens showed essentially no mutational event (Example I). Absence of hypermutation during somatic transgene immunization is expected since the transgene lacks the transmembrane domain, making it difficult for antigen or anti-immunoglobulin antibodies to exert selective pressure on the B cells transduced *in vivo*.

Intraspleen inoculation of DNA containing viral enhancer-promoter sequences failed to induce an immune response (Raz et al., *supra*, 1994), and most studies on intramuscular inoculation have indicated that multiple DNA injections are needed for a measurable and sustained antibody response (reviewed in (Whalen and Davis, *supra*, 1995; Whalen et al., Ann. N. Y. Acad. Sci. 772:64-76 (1995)). Therefore, it is likely that transfection of spleen B lymphocytes with an immunoglobulin H chain gene under the control of tissue-specific promoter and enhancer elements was crucial to successful initiating and sustaining of the immune response.

As disclosed herein, an endogenous antigen, the transgene H chain product, secreted in low amounts (15-30 ng/ml) is per se immunogenic rather than tolerogenic (see

Example I). This is in agreement with earlier experiments (Reth et al., Nature 290:257-259 (1981)) showing the enhancing effect of minute amounts (10 ng) of isologous anti-idiotypic response following antigen immunization. The serum concentration of transgene H chain immunoglobulins detected in mice is compatible with the estimated minimum antibody concentration (20 ng/ml) necessary to form antigen:antibody complexes (Langman and Cohn, Mol. Immunol. 24:675-697 (1987)). Protein antigens are more immunogenic when administered in aggregated form (Dresser, Immunology 5:378-388 (1962); Golub and Weigle, J. Immunol. 102:309-315 (1969)) or complexed with antibodies (Klaus, Nature 278:354-355 (1979a); Klaus, Adv. Exp. Med. Biol. 114:289-294 (1979b)), as this results in a greater uptake by macrophages and dendritic cells (Tew et al., Immunological Rev. 53:175-201 (1980)). Consequently, the continuous production of anti-immunoglobulin antibodies was likely sustained by immune complexes (H chain transgene immunoglobulins/anti-immunoglobulins) captured and stored by follicular dendritic cells, and subsequently presented to T cells, which are required for anti-immunoglobulin responses *in vivo* (Coulie and Van Snick, J. Exp. Med. 161:88-97 (1985); Nemazee, J. Exp. Med. 161:242-256 (1985)).

The results described herein in Example I indicate similarities between the immune response triggered by intraspleen inoculation of DNA and immunity against foreign antigens. Antigens that invade an organism through the skin are sequestered by dendritic cells (Langherans cells) and vehicled to secondary lymphoid organs (lymph nodes and spleen). Antigens that invade the organism via the blood are trapped in the white pulp of the spleen where dendritic cells of the

follicular zone capture, process and present antigen to B and T lymphocytes (Anderson, in Structure and Organization of the Lymphatic System, Oppenheim and Shevach, eds., Oxford University Press, New York (1990)).

5 Clonal activation and expansion of B and T cells begin following this initial step. STI initiates the process of immunity directly within the anatomical structures which constitute the afferent point of foreign antigen during a conventional immune response. The fact that the
10 transgene is harbored in B lymphocytes (Xiong et al., Proc. Natl. Acad. Sci. USA 94:6352-6357 (1997)) insures a protracted synthesis and secretion of transgenic immunoglobulins in quantities and form sufficient for the initiation of immunity and the establishment of
15 immunologic memory.

The type of immunogenic stimulus offered by somatic transgene immunization can persist in the organism as long as B lymphocytes harboring the transgene
20 live and secrete the transgene product. In the mouse, spleen B lymphocytes are distinguished into short-lived (3-4 days) (Rocha et al., Eur. J. Immunol. 20:1697-1708 (1990)), representing approximately one third, and long-lived (> 6 weeks in the resting state) (Forster and
25 Rajewsky, Proc. Natl. Acad. Sci. USA 87:4781-4784 (1990)), representing approximately two thirds of the total population (Osmond, Curr. Op. Immunol. 3:179-185 (1991)). After a single intraspleen inoculation, the transgene persisted functionally for four months. Thus,
30 it appears that the cells transfected *in vivo* were long-lived B lymphocytes (see Example II). The transgene can persist in the host throughout the life span of the host B cell to disappear when the B cell dies. This, together with the "depot effect" played by follicular dendritic
35 cells, may be critical in the induction and maintenance

of memory B cells whose half-life in the absence of antigen is estimated in the order of 2-3 weeks (Gray and Skarvall, Nature 336:70-73 (1988)).

5 B lymphocytes are generated in the bone marrow and localize in secondary lymphoid organs and in the blood throughout life (Osmond, Immunol. Rev. 93:103-124 (1986); Rajewsky, Curr. Opin. Immunol. 4:171-176 (1992); Rolink Melchers Curr. Opin. Immunol. 5:207-217 (1993)).

10 Upon activation by antigen, a B cell can produce between 1×10^3 and 8×10^4 molecules of immunoglobulin(Ig)/cell/sec (Jerne, Immunol. Rev. 79:5-24(1984); Langman and Cohn, *supra*, 1987). Consequently, B cells are formidable minifactories of proteins in mammals. B lymphocytes can
15 also present antigen to T lymphocytes: (i) antigens internalized via their membrane Ig receptor (Lanzavecchia, *supra*, 1985), and (ii) peptides of secretory proteins including their own Ig (Weiss and Bogen, Proc. Natl. Acad. Sci. USA 86:282-286 (1989);
20 Billetta et al., Eur. J. Immunol. 25:776-783 (1995)). Because of these properties, B lymphocytes constitute an ideal substrate for strategies of gene targeting and immunization with plasmid DNA.

25 The goal of immunization against pathogens (vaccination) is to generate immunity that confers protection (Salk and Salk, Science 195:834-847 (1977)). In many instances, both humoral and cellular responses, together with the induction of immunologic memory from
30 the first injection, are needed. Furthermore, the antigenicity and immunogenicity of a candidate vaccine may be enhanced if the relevant epitopes are expressed in an appropriate three-dimensional conformation (Zanetti et al., Immunol. Today 8:18-25 (1987)).

5 The methods of the invention are useful for
stimulating an antibody response. Hypervariable loops of
immunoglobulins (Ig) can be used to express discrete
peptide sequences of antigens, antigenized antibodies
10 (Zanetti, *supra*, 1992). These antibodies serve as
immunogens that focus the immune response on specific B
or T cell epitopes (Zanetti et al., *supra*, 1992). Unlike
vaccines produced by synthetic approaches, antigenized
antibodies (i) express epitopes with intrinsic tri-
15 dimensional conformation for immunogenicity at the B cell
level (Sollazzo et al., *supra*, 1990a; Billetta et al.,
supra, 1991; Lanza et al., *supra*, 1993; Zaghouani et al.,
supra, 1995), (ii) target antigen-presenting cells via
the Fc receptor, hence maximizing antigen presentation by
20 class II MHC molecules (Zaghouani et al., *supra*, 1993),
and (iii) provide B cells with a continuous source of
antigenic peptides for presentation in class I MHC
molecules (Billetta et al., *supra*, 1995). H chain genes
under the control of Ig promoter and enhancer elements
25 inoculated into the spleen as plasmid DNA are
incorporated into B lymphocytes and initiate immunity
against transgene H chain Ig secreted in the serum (see
Examples I and II).

25 The results described herein illustrate the use
of STI to induce antigen-specific immunity (see Example
III). Immunity against three repeats of the hydrophilic
tetrapeptide sequence Asn-Ala-Asn-Pro (NANP), a B-cell
epitope expressed on the surface of *Plasmodium falciparum*
30 malaria sporozoites, engineered in the CDR3 of a H chain
gene. This amino acid sequence is present in multiple
tandem repeats in the central portion of the
circumsporozoite (CS) protein (Zavala et al., Science
228:1436-1440 (1985)). Antibodies against this epitope
35 develop in people living in endemic areas for malaria

(Zavala et al., *supra*, 1985; Nardin et al., Science 206:597-601 (1979)) as well as in volunteers vaccinated with irradiated sporozoites (Clyde et al., Am. J. Med. Sci. 266:398-403 (1973); Calle et al., J. Immunol. 149:2695-2701 (1992); Egan et al., Am. J. Trop. Med. Hyg. 49:166-173 (1993)). This is the only malaria antigen that, when used as a subunit vaccine, has conferred protection against experimental sporozoite challenge in human volunteers (Ballou et al., Lancet 1:1277-1281 (1987); Herrington et al., Nature 328:257-259 (1987); Edelman et al., J. Infect. Dis. 168:1066-1070 (1993)). The results disclosed herein show that STI allows the immune system to be programmed for the production of anti-parasite antibodies and the establishment of immunologic memory against the *P. falciparum* parasite (see Example III).

As disclosed herein in Example III, immunity against the human malaria parasite *Plasmodium falciparum* was induced using somatic transgene immunization, a method to effectively target B lymphocytes *in vivo*. A single inoculation of plasmid DNA containing an immunoglobulin heavy chain gene coding in the CDR3 for three repeats of, the sequence Asn-Ala-Asn-Pro (NANP), a B-cell epitope of *P. falciparum* sporozoites, induced antibodies against NANP in all mice. Immunologic memory was also established as revealed by booster with an antibody antigenized with the NANP peptide administered in adjuvant or through challenge with *P. falciparum* sporozoites. During the primary response, anti-NANP antibodies were primarily IgM and IgG2a. After booster, all antibody isotypes, including IgG1, developed readily. Thus, immunity to a parasite antigen can be induced by exploiting mechanisms in which B lymphocytes are both the source of the immunogen as well as the effector mechanism

of immunity. The results disclosed herein indicate that somatic transgene immunization is an effective approach for vaccination against foreign pathogens.

5 The methods of the invention can be used to stimulate a T cell response such as a CD4 T cell response and/or a CD8 T cell response. Hypervariable loops of immunoglobulins (Ig) can be used to express discrete peptide sequences of antigens, antigenized antibodies
10 (Zanetti, *supra*, 1992). These can be the amino acid sequences of epitopes that induce specific responses in T lymphocytes, CD4⁺ and CD8⁺.

15 Encounter with antigen and sufficient co-stimulation transform naive T cells into effector cells, accompanied by clonal expansion and expression of cytokines other than IL-2. Early effector Th cells (Th0 cells) produce both Th1 (IFN- γ) and Th2 (IL-4, IL-5) cytokines . As differentiation progresses through
20 pressure by environmental cytokines and antigen, T cells can be polarized towards the Th1 or Th2 phenotype , but in the absence of such selective pressure remain uncommitted and retain production of all cytokines .

25 As disclosed herein in Example VIII, cellular immune responses were analyzed *in vivo* after a single intraspleen inoculation of DNA coding for a 12 residue Th cell determinant associated with a 12 residue B cell epitope, a process termed somatic transgene immunization.
30 As disclosed herein, CD4 T cells are readily activated and produce IL-2, IFN- γ and IL-4, characteristics of an uncommitted phenotype. Although originating in the spleen, T cell responsiveness was found to spread immediately and with similar characteristics to all lymph
35 nodes in the body. A single inoculation was also

effective in establishing long term immunologic memory as determined by limiting dilution analysis, with memory T cells displaying a cytokine profile different from primary effector T cells.

5 These studies provide evidence that by initiating immunity directly in secondary lymphoid organs, one generates an immune response with characteristics that differ from those using vaccines of
10 conventional DNA or protein in adjuvant administered in peripheral sites. Somatic transgene immunization can therefore be used to induce Th cell responsiveness *in vivo*.

15 The methods of the invention are also useful for stimulating an antibody response in combination with a T cell response such as a CD4 T cell response. Such a combined response can be termed associative recognition. Inclusion of multiple epitopes from the same antigen or
20 combination of epitopes with different immunogenic function in the same molecule can be used in nucleic acid molecules of the invention. For instance, the antibody response to protein antigens requires the cooperation between B cells and T helper (Th) cells (Mitchison, Eur. J. Immunol. 1:18-27 (1971)) with optimal conditions
25 occurring when B and Th cells are specific for different determinants on the same molecule (associative recognition). Based on this principle, synthetic peptide vaccines (Herrington et al., *supra*, 1987; Tam and Lu, Proc. Natl. Acad. Sci. USA 86:9084-9088 (1989); Tam et
30 al., J. Exp. Med. 171:299-306 (1990); Munesinghe et al., *supra*, 1991) or recombinant subunit vaccines (Ballou et al., *supra*, 1987; Herrington et al. Vaccine 10:841-846 (1992)) designed and tested in recent years included both
35 B and Th cell epitopes.

As disclosed herein, an antigenized antibody gene coding for two distinct 12 amino acid long peptides representing a B (Zavala et al., *supra*, 1985) and a Th (Munesinghe et al., *supra*, 1991; Nardin et al., *Science* 246:1603-1606 (1989) cell epitope of the circumsporozoite (CS) protein of *P. falciparum* malaria parasite were expressed and tested. Engineering of the CDR3 and the CDR2 of the same V_H domain did not significantly affect secretion *in vivo* of the antigenized antibody molecules. Mice inoculated into the spleen with this gene mounted an antibody response against the B cell epitope higher than mice receiving the gene coding for the B cell epitope only. *In vitro* studies established that the two epitope were independently immunogenic *in vivo* (see Example IV).

The methods of the invention can similarly by used for associative recognition to stimulate a Th/Th response. While the importance of associative (linked) recognition events in the development of an adaptive immune response are universally accepted, it is not known yet whether or not the same concept applies to a cooperative interaction between Th cell epitopes on the same molecule. Experiments using an antigenized antibody gene in the context of STI revealed that this to be the case (see Figure 35 and Example X).

As disclosed herein, two Th cell epitope expressed in the CDR2 and CDR3 of the same gene, respectively, were independently immunogenic *in vivo* (Figure 36 and Example X).

The structure of V_H domains of Ig has been extensively investigated and it is generally accepted that, while the β -strands of framework regions are

conserved and maintain the surface topology between V_H and V_L (Alzari et al., Ann. Rev. Immunol. 6:555-580 (1988), CDR loops interconnecting two β -strands vary extensively in amino acid composition (Kabat et al., Proteins of Immunological Interest, U.S. Department of Health and Human Services, Bethesda MD (1987)), hence creating a vast array of binding sites for antigens. Differences exist, however, between the CDR3 and CDR2 with respect to the overall structure of the V_H domain. While CDR3 loops can vary considerably in length (Kabat et al., *supra*, 1987), CDR2 loops are grouped in few canonical structures (Chothia et al., Nature 342:877-883 (1989); Chothia and Lesk, J. Mol. Biol. 196:901-917 (1987), possessing key "signature" residues and conserved length to ensure V_H/V_L scaffolding. The results disclosed herein in Example IV stand as an exception to the canonical structure paradigm. As demonstrated, a drastic structural change (both in length and amino acid composition) of the CDR2 loop did not affect folding and pairing of the antigenized VH domain with VL *in vivo* (see Example IV).

The ability to manipulate Ig V region genes and express multiple heterologous peptides in the CDRs open new possibilities in the design of molecules of complex, predetermined antigen specificity and/or complementary immunogenic function, for example, B/Th, Th/Th or Th/CTL epitopes, depending on the desired effect, for vaccination purposes. The demonstration that the antigenized H chain genes can be easily expressed *in vitro* and can be used *in vivo* points to new strategies of vaccination. Because antigenized H chain genes elicit an immune response *in vivo*, multiple antigen peptides can be used in various combinations to optimize immunogenicity.

5 The methods of the invention can be used to
establish immunological memory. Long-lasting humoral
immunity after DNA inoculation has already been
documented in few instances (Raz et al., *supra*, 1994;
Davis et al., Vaccine 14:910-915 (1996)), although in
many reports multiple DNA injections seem to be required
(Sedegah et al., *supra*, 1994; Xiang et al., *supra*, 1994;
Anderson et al., *supra*, 1996). As disclosed herein, the
induction and persistence of specific antibodies was
10 observed at high titers for 18 weeks and at a lower
titers for two years (see Example V). These results
indicate that specific B cell clonotypes are more easily
activated by a prolonged release in the lymphoid
microenvironment of minute amounts of the endogenously-
15 synthesized antigen rather than through administration of
antigen in adjuvant.

20 A key feature of STI is the establishment of
persistent immunologic memory. Booster injection of the
Y1NANP protein in adjuvant 6, 30 or 104 weeks after
priming resulted in a *bona fide* anamnestic response.
Specific memory exists when mice are challenged with *P.*
falciparum parasites 6 weeks after priming (see Example
III). These results indicate that presentation of the
25 NANP epitope through the Ig H chain gene product mimics
immunization with the native antigen and that, in turn,
NANP-reactive B cells primed via STI are of sufficient
affinity to recognize the NANP epitope present in its
natural molecular environment at the surface of the
30 parasite.

The observation that a single inoculation of
appropriately formulated plasmid DNA establishes
persistent immunologic memory is reminiscent of the
35 earlier observation that the immunologic memory that

follows a single dose of a suitably potent vaccine increases over time and persists thereafter (Salk, Rev. Infect. Dis., S444-450 (1984)). Similarly, STI triggers, and subsequently maintains, a state of durable immunity and possibly irreversible immunologic memory without the periodic need for reinforcement. Therefore, STI is applicable to those pathogens or conditions which require memory-dependent immunity (Salk and Zanetti, in Progress in Vaccinology, Vol. 2, Talwar, ed., Springer-Verlag, New York (1989)).

Specific B cell memory was present long after disappearance of both the transgene product (3-4 weeks) or the transgene itself from the organ of inoculation (~3 months), and was not impeded by circulating antibodies to the transgene product. Conventional views suggested that B cell memory originates from one of two alternative possibilities. One possibility is that the continuous presence of antigen, in this case the transgene product, maintains specific B cells under constant antigen selection and stimulation. The other possibility is that once established, memory B cells hide in lymphoid organs until antigen is re-encountered (Ahmed and Gray, Science 272:54- 60 (1996)). In the case of STI, immunologic memory appears to be independent of the persistence of the transgene product or even the existence of the transgene in the organ of inoculation. It is possible that, since it is known that follicular dendritic cells play a role as antigen depot (Tew et al., *supra*, 1980) and this may be relevant to the maintenance of immunologic memory, sufficient amounts of transgene H chain Ig, alone or in the form of complex, could be stored in follicular dendritic cells for a time long enough to provide the stimulus for the maintenance of memory B cells.

Regulation of Ig isotype switch is a complex phenomenon mainly under control of cytokines secreted by CD4⁺ T cells (TH1 and TH2) activated during presentation of antigen peptides by dendritic cells (DC), B lymphocytes and macrophages. TH1 cells secrete IL-2 and IFN- γ , whereas TH2 cells secrete IL-4, IL-5 and IL-10 (Harriman et al., Ann. Rev. Immunol. 11:361-384 (1993)). Although Ig isotype switch is conveniently and clearly regulated *in vitro* by cytokines (Snapper et al., J. Immunol. 140:2121-2127 (1988); Isakson et al., J. Exp. Med. 155:734-748 (1982); Coffman et al., J. Immunol. 136:4538-4541 (1986); Snapper et al., J. Exp. Med. 167:183-196 (1988)), *in vivo* regulation using anti-cytokine antibodies is less clear (Finkelman et al., Int. Immunol. 3:599-607 (1991)). Reasons for this discrepancy could lie on the fact that, *in vivo*, CD4⁺ T cells do not segregate into categories with distinct TH1/TH2 characteristics (Kelso and Gough, Proc. Natl. Acad. Sci. USA 85:9189-9193 (1988); Paliard et al., J. Immunol. 141:849-855 (1988)). Moreover, isotype switch may be influenced by relative concentrations of cytokines reflecting the avidity of cognate T-B interaction and previous T cell activation (Croft and Swain, J. Immunol. 147:3679-3689 (1991)). No previous information has been available concerning the possible influence on isotype switch *in vivo* by other cell types.

As disclosed herein, the cellular requirements potentially involved in the IgM to IgG1 switch *in vivo* during priming induced by somatic transgene immunization was investigated (see Example VI).

Inoculation of plasmid DNA in the form of a chimeric gene coding for GM-CSF was able to drive IgG1 class switch readily after priming. Since GM-CSF

activates cells of the dendritic lineage, the role of dendritic cells (DC) in regulating the IgM to IgG1 switch was investigated. Bone marrow chimeras were constructed from mice carrying the null mutation for the *relB* member of the NF- γ B/Rel family since these mice lack bone-marrow derived mature DC. *RelB* (-/-) mice and (-/-) bone marrow chimeras inoculated with DNA/GM-CSF did not produce IgG1 antibodies during the primary immune response. Since *relB* (-/-) bone-marrow chimeras lack DC of donor origin but possess resident follicular dendritic cells (FDC), these results suggest that Ig class switch *in vivo* is regulated by the function of interdigitating dendritic cells (IDC). Thus, IDC appears contribute to the qualitative aspects of the emerging immune response.

As disclosed herein in Example VI, isotype switch is likely controlled by T cell-derived cytokines in B cell foci on the edge of T zones of the spleen following the interaction of T cells with B cells and DC. Activation of DC using DNA chimerized with the GM-CSF coding region resulted in a prompt IgM to IgG1 switch, indicating that *in vivo*, DC are involved in a cellular network which regulates the IgM to IgG1 switch.

The results disclosed herein in Example VI indicate mechanisms of the immune response *in vivo*. By targeting anatomical structures normally constituting the afferent point of the immune response to foreign pathogens and by injecting DNA of increasing complexity for enhanced immunogenicity and adjuvanticity, it became possible to assign a role to bone marrow derived IDC in controlling the IgM to IgG1 switch (see Example VI). This effect is presumably secondary to other properties of DC such as activation of naive T cells and strengthening the avidity of T-B cell interactions

(Steinman, Annu. Rev. Immunol. 9:271-296 (1991)). The results disclosed herein indicated that *in vivo* influence on isotype switch was noted only with respect to IgG1.

5 Among natural immunologic adjuvants, GM-CSF was shown to increase the potency of immunization against tumor cells and protein antigens. In the course of studies on STI it was realized that GM-CSF affects antibody-mediated memory responses. As disclosed herein
10 in Example VII, GM-CSF given at priming as a DNA/GM-CSF chimeric vaccine enhances the magnitude of the anamnestic response irrespective of the form of antigen used subsequently in the booster immunization. Using mice lacking bone marrow-derived DC, it was also determined
15 that the enhancing effect is not strictly dependent on these cells. These results expand our understanding on the potential utility of GM-CSF *in vivo* as a modulator of the immune response and immunologic memory induced via STI (see Example VII).

20 GM-CSF has been used in a variety of systems as immunological adjuvant to increase the potency of immunization. Early reports indicated that tumor cells transfected with GM-CSF induce potent and protective
25 anti-tumor responses (Dranoff et al., *supra*, 1993; Levitsky et al., *supra*, 1996). Similarly, an immunoglobulin (Ig) idiotype /GM-CSF chimeric protein was shown to induce higher IgG1 anti-idiotypic response than idiotype alone and to protect against the growth of B
30 lymphoma cells *in vivo* (Tao and Levy, *supra*, 1993; Chen et al., J. Immunol. 153:4775-4787 (1994)). GM-CSF has also been used to enhance immune responses to DNA vaccination (Xiang and Ertl, *supra*, 1995). In one
35 instance, mice inoculated with an idiotype/GM-CSF chimeric gene were found to resist growth of B lymphoma

cells better than mice vaccinated with the idiotype gene without GM-CSF (Syrengelas et al., *supra*, 1996).

As disclosed herein, priming with an antigenized antibody /GM-CSF DNA vaccine enhances the magnitude of the anamnestic response against a defined dodecapeptide B cell determinant irrespective of the form of antigen used in the booster immunization (Example VII). Moreover, experiments in mice lacking bone marrow-derived DC indicate that the enhancing effect of GM-CSF is not strictly dependent on these cells. The results disclosed herein define a role for the activity of GM-CSF *in vivo* as a modulator of the immune response, including immunologic memory.

As disclosed herein in Example VIII, cellular immune responses were analyzed *in vivo* after a single intraspleen inoculation of DNA coding for a 12 residue Th cell determinant associated with a 12 residue B cell epitope, a process termed somatic transgene immunization. As disclosed herein, CD4 T cells are readily activated and produce IL-2, IFN- γ and IL-4, characteristics of an uncommitted phenotype. Although originating in the spleen, T cell responsiveness was found to spread immediately and with similar characteristics to all lymph nodes in the body. A single inoculation was also effective in establishing long term immunologic memory as determined by limiting dilution analysis, with memory T cells displaying a cytokine profile different from primary effector T cells. These studies provide evidence that by initiating immunity directly in secondary lymphoid organs, one generates an immune response with characteristics that differ from those using vaccines of conventional DNA or protein in adjuvant administered in peripheral sites.

The methods of the invention can be used to administer a nucleic acid molecule encoding one or more heterologous epitopes to a lymphoid tissue. Administration to a lymphoid tissue provides targeting of the nucleic acid molecule to a lymphoid cell. Therefore, the methods of the invention can also be used to administer a nucleic acid molecule to a lymphoid cell. Administration of a nucleic acid molecule to a lymphoid cell allows the protein expression capabilities of the lymphoid cell to be used to express the epitope against which the stimulation of an immune response is desired.

A nucleic acid molecule of the invention can be targeted to a lymphoid cell. The lymphoid cell can be targeted *in vivo* or *ex vivo*. For example, as described above, a nucleic acid molecule can be administered to an individual *in vivo* to target a lymphoid cell. For example, the nucleic acid molecule can be administered to a lymphoid tissue, resulting in targeting of hematopoietic cells, including a lymphoid cell, in the lymphoid tissue. However, it is understood that a nucleic acid molecule of the invention can be administered by any method or route that results in targeting of a hematopoietic cell such as a lymphoid cell for expression of the epitope encoded by the nucleic acid molecule.

A nucleic acid molecule of the invention can also be administered *ex vivo*. For example, hematopoietic cells, including lymphoid cells, can be obtained from an individual or from an immunologically compatible individual, and a nucleic acid molecule of the invention can be administered to these cells *ex vivo*. Methods of administering nucleic acid molecules to cells *ex vivo* are well known in the art and include, for example, calcium

phosphate precipitation and electroporation (see, for example, Sambrook et al., Molecular Cloning a Laboratory Manual Cold Spring Harbor Press (1989); Ausubel et al., Current Protocol in Molecular Biology, Wiley & Sons (1998)) These lymphoid cells, which now contain the nucleic acid molecule and express the encoded epitopes, can then be administered to an individual. The lymphoid cells expressing the epitopes can then stimulate an immune response.

Methods of introducing a gene of interest into a whole organism are well known in the art (see, for example, Kay et al., Proc. Natl. Acad. Sci. USA, 89:89-93 (1993); Chowdbury et al., Science, 254:1802-1805 (1991); Grossman et al., Nature Genetics, 6:335-341 (1994); Malech et al., Proc. Natl. Acad. Sci. USA, 94:12133-12138 (1997)).

The invention additionally provides methods of treating a condition by administering a nucleic acid molecule of the invention, where the nucleic acid molecule is targeted to a hematopoietic cell. The administration of a nucleic acid molecule expressing an epitope to stimulate an immune response is useful for treating a condition as described above. The methods of the invention for treating a condition by targeting a hematopoietic cell are useful when a B cell, T cell or dendritic cell. The methods of the invention for treating a condition are particularly useful when a B cell is targeted.

The invention further provides methods of treating a condition by administering a nucleic acid molecule comprising a hematopoietic cell expression element operationally linked to a nucleic acid molecule

encoding one or more heterologous polypeptides, where the nucleic acid molecule is targeted to a hematopoietic cell. The targeted hematopoietic cells serve to express a heterologous polypeptide to treat a condition. The methods of the invention are advantageous for administering a therapeutic polypeptide to treat a condition. The methods of the invention can be used, for example, to express a hormone, cytokine, clotting factor or immunoglobulin. For example, if an individual has a condition for which an increase in expression of a hormone or cytokine would be beneficial, such an individual can be treated by administration of a nucleic acid molecule expressing a hormone or cytokine polypeptide. For example, an individual having a condition characterized by immunodeficiency can be treated by administering a cytokine such as IL-2 or INF- γ , or other cytokine, as disclosed herein, or by administering an immunoglobulin. Similarly, an individual suffering from a condition such as hemophilia can be treated, for example, by administering a nucleic acid molecule encoding a clotting factor such as factor VIII or factor IX. One skilled in the art can readily determine an appropriate polypeptide to express for treating a given condition.

The methods of the invention can be used to treat a condition by expressing a wide variety of disease-associated gene products of interest, which can be employed to treat or prevent the disease of interest. For example, and by way of illustration only, the genes can encode enzymes, hormones, cytokines, antigens, antibodies, clotting factors, anti-sense RNA, regulatory proteins, ribozymes, fusion proteins and the like. The methods can thus be used to supply a therapeutic protein such as Factor VIII, Factor IX, Factor VII,

erythropoietin (U.S. Patent No. 4,703,008), alpha-1-antitrypsin, calcitonin, growth hormone, insulin, low density lipoprotein, apolipoprotein E, IL-2 receptor and its antagonists, superoxide dismutase, immune response
5 modifiers, parathyroid hormone, the interferons (IFN alpha, beta or gamma), nerve growth factors, glucocerebrosidase, colony stimulating factor, interleukins (IL) 1 to 15, granulocyte colony stimulating factor (G-CSF), granulocyte, macrophage-colony
10 stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CFS), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), adenosine deaminase, insulin-like growth factors (IGF-1 and IGF-2), megakaryocyte promoting ligand (MPL, or thrombopoietin).
15 The therapeutic polypeptides can be useful, for example, for the treatment and prevention of genetic disorders such as coagulation factor disorders, glycogen storage disease, and alpha-1-antitrypsin deficiency. The methods of the invention can also be used to express ligands of
20 adhesion molecules such as integrins, for example, to block adhesion function such as angiogenesis.

The invention also relates to pharmaceutical compositions comprising a pharmaceutically acceptable
25 carrier and a nucleic acid molecule of the invention. The methods of the invention can therefore utilize pharmaceutical composition comprising a nucleic acid molecule of the invention encoding an epitope. Pharmaceutically acceptable carriers are well known in
30 the art and include aqueous or non-aqueous solutions, suspensions and emulsions, including physiologically buffered saline, alcohol/aqueous solutions or other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize the nucleic acid molecules to be administered or increase the absorption of the nucleic acid molecules. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants such as ascorbic acid or glutathione, chelating agents, low molecular weight polypeptides, antimicrobial agents, inert gases or other stabilizers or excipients. Nucleic acid molecules can additionally be complexed with other components such as peptides, polypeptides and carbohydrates. Nucleic acid molecules can also be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector. As described above, the route of administration will generally be direct injection into a secondary lymphoid tissue.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Somatic Transgene Immunization with DNA Encoding an Immunoglobulin Heavy Chain

This example describes immunization with plasmid DNA by direct injection into the spleen.

The methods for preparation of plasmid DNA and immunization by injection into the spleen are described below (Gerloni et al., DNA Cell Biol. 16:611-625 (1997)).

5 Eight to ten week old C57BL/6 (H-2^b) mice were purchased from the Jackson Laboratories (Bar Harbor, ME) or Harlan Sprague-Dawley (San Diego, CA). Mice were maintained in the animal facility of the University of California, San Diego, throughout the duration of the
10 experiments.

 Four plasmid vectors utilized for *in vivo* inoculation. γ 1WT defines plasmid pNeoy₁62 (Sollazzo et al., Eur. J. Immunol. 19:453-457 (1989)) which contains
15 the 2.3 Kb EcoRI DNA fragment carrying the genomic murine V_H⁶² rearrangement at the unique EcoRI site of the pNyl vector (Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851-6855 (1984)). γ 1WT encodes a genomic human γ 1 gene and contains a neomycin resistance gene under the
20 control of the SV40 promoter for the selection of stable transformant cells. γ 1WT-TAC is a modified version of the γ 1WT plasmid in which the three base pairs of TAC were introduced by oligonucleotide-directed mutagenesis in the CDR3 as a genetic marker. This was used uniquely
25 to study gene integration. γ 1NANP is a modified version of the γ 1WT plasmid where the third hypervariable region was modified to encode three repeats of the tetrapeptide Asn-Ala-Asn-Pro (NANP) sequence (et al., Prot. Engng. 4:215-220 (1990a)). This plasmid was used for mRNA
30 analysis of the V_H region. pSV2Neo is the original plasmid forming the backbone of the pNeoy1 vector without the human γ 1 C region gene (Mulligan and Berg, Proc. Natl. Acad. Sci. USA 78:2072-2076 (1981)). This plasmid was used as a control in the immunization experiments.
35 These three plasmids are illustrated in Figure 1. γ 2bV_H³⁶⁻

⁶⁵ defines a plasmid containing the V_H^{36-65} gene (Sharon et al., J. Immunol. 142:596-601 (1989)) joined to a murine $\gamma 2b$ C region gene. All plasmid DNAs were prepared from DH5 γ *Escherichia coli* according to standard procedures (Sambrook et al., Molecular Cloning. A Laboratory Manual., 2nd ed., Cold Spring Harbor Laboratory Press (1989)). The purity of the DNA was monitored using the following equation: $\%N = (11.1R - 6.32) / (2.16 - R)$ where $R = 260nm/280nm$, $\%N = \% \text{ of Nucleic Acid}$ (Glasel, BioTechniques 18:62-63 (1995)).

Engineered antibodies $\gamma 1WT$, $\gamma 2bWT$ and $\gamma 2bV_H^{36-65}$ were prepared by electroporation (Billetta and Zanetti, ImmunoMethods 1:41-51 (1992) of the $\gamma 1WT$, $\gamma 2bWT$ (Sollazzo et al., *supra*, 1989) and $\gamma 2bV_H^{36-65}$ (Sharon et al., *supra*, 1989) plasmid DNAs into the murine J558L cell line, an H chain-defective variant of the J558 myeloma cell (Morrison, Science 229:1202-1207 (1985)). Transfectant cells were screened for immunoglobulin production by ELISA and the immunoglobulins were purified by affinity chromatography on a Protein A column (Billetta and Zanetti, *supra*, 1992).

Mice were inoculated with 100 μg of plasmid DNA per inoculation. All DNA inoculations were done in the absence of immunological adjuvants. Four basic routes of inoculation were used. a) Intramuscular. The plasmid DNA was injected in the quadriceps in 30 μl volume in sterile saline. Thereafter, mice received three booster injections at weekly intervals for a total of four injections. b) Subcutaneous. The plasmid DNA was injected in the back in 25-50 μl volume of sterile saline. Thereafter, mice received three booster injections at weekly intervals for a total of four injections. c) Intravenous. The plasmid DNA was injected

in 50-100 μ l volume of sterile saline solution via the tail vein. Thereafter, mice received three booster injections at weekly intervals for a total of four injections. d) Intraspleen. The plasmid DNA was injected in 30 μ l volume of sterile saline solution. For the intraspleen inoculation mice were anesthetized with a cocktail of Ketamine, Xylazine and Acepromazine. Anesthetized mice were shaved locally and a small incision (3-4 mm) was made with a scalpel in the upper left portion of the abdomen under the diaphragmatic line. By pulling gently the fat of the ventral part of the spleen the organ was exposed through the small incision and injected with DNA using a 28 gauge needle. The abdomen was immediately sutured using 4-0 sterile, non-absorbable surgical suture thread. Mice inoculated via this route were injected only once. In one experiment (Table 1) mice received three booster injections of 100 γ g of DNA intravenously. Mice were bled via the retro-orbital route on day 0 and subsequently at 7 days intervals.

Mice were immunized with affinity-purified γ 1WT protein adsorbed on alum (50 μ g per mouse) intraperitoneally. Mice that were boosted with the γ 1WT protein received 50 μ g of the protein emulsified in incomplete Freund's adjuvant subcutaneously.

The detection of antibodies to γ 1WT, γ 2bWT and γ 2bV_H³⁶⁻⁶⁵ proteins was performed by direct ELISA on 96-well polyvinyl microtiter plates (Dynatech; Gentilly VA) coated (2.5 μ g/ml) by drying at 37 °C. After coating wells were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.3. Wells were incubated overnight at 4°C with mice sera diluted in 1% BSA-PBS, containing 0.05 % Tween-20 (PBSA). The bound

antibodies were revealed using a goat antibody to mouse γ -globulins absorbed with human γ -globulins and conjugated with horseradish peroxidase (HP) (Pierce; St. Louis MO). The bound peroxidase was revealed by adding
5 o-phenylenediamine dihydrochloride and H_2O_2 . Plates were read after 30 minutes in a micro-plate reader (Vmax, Molecular Devices) at 492 nm. Tests were done in duplicate.

10 The presence of γ 1WT H chain transgene polypeptide in the serum of mice was detected by ELISA capture assay (Billetta and Zanetti, *supra*, 1992). Briefly, 1:10 dilution of individual mouse sera in PBSA were incubated on 96-well plate coated with a goat
15 antibody to human γ -globulin (10 μ g/ml). To determine the concentration of the immunoglobulin H chain transgene product in the serum O.D. values were plotted against a standard curve constructed with known amount of human γ -globulins diluted in PBSA containing 10% normal mouse
20 serum. The bound antibodies were revealed using a HP-conjugated goat antibody to human γ -globulins (H chain specific) absorbed with murine immunoglobulins (Sigma; St. Louis MO). The bound peroxidase activity was revealed by adding o-phenylenediamine dihydrochloride and
25 H_2O_2 . The test was continued as detailed above.

The kinetics of synthesis of the transgenic mRNA was studied by reverse RT-PCR in the spleen of mice inoculated via the intrasplenic route. Briefly, spleens
30 were harvested 3, 10, 20, 127 days after the initial inoculation, frozen at -170°C , and the cells were prepared by gentle teasing. Typically, PolyA⁺ mRNA was isolated from 11 mg of spleen tissue using the Micro-FastTrack mRNA isolation kit (Invitrogen; San Diego CA).
35 cDNA was synthesized by reverse transcription using the

cDNA Cycle Kit (Invitrogen, San Diego). Specific PCR primers for the murine V_H , TTCGATGTCCATACCATGAGAGTA and TTCAGCACCTACTATCCAGACACT and the human $\gamma 1$ C region genes TTCCTCTTCTGCGTGTAGTGGTTG and TTCATAATGCCAAGACAAAGCCGC were designed using Oligo 4.0 Primer Analysis Software (NBI, Inc.; Plymouth MN) from the sequences of the murine V_H^{62} and human $\gamma 1$ C region genes obtained from the National Center for Biotechnology Information (NCBI) GenBank (Bethesda MD). Primers for the β -actin gene (Figure 4) were used to control for the extraction and reverse transcription of spleen mRNA. The PCR reaction with *Taq* polymerase (Gibco BRL; Gaithersburg, MD) was performed according to the following conditions: 1 min at 92°C followed by 2 min at 40°C and 3 min at 72°C. This cycle was repeated 30 times. cDNA amplification and size determination (V_H^{62} = 198 bp; human $\gamma 1$ C region gene = 466 bp) were checked by electrophoresis using 4% NuSieve 3:1 agarose gel (FMC; Rockland ME).

All DNAs analyzed by Southern blot were separated by electrophoresis on a 1% agarose gel and transferred to Hybond-N nylon membrane (Amersham; Cleveland, OH). Blots were hybridized with oligonucleotide probes labeled using T4 polynucleotide kinase forward reaction in presence of (γ - 32 P ATP).

For extraction of genomic DNA from spleen tissue and genomic DNA sequencing, spleens were harvested 17 days after DNA inoculation, frozen at -170°C and the cells were prepared by tissue grinding in liquid nitrogen. Typically the genomic DNA was extracted from 10 mg of spleen tissue using the QIAamp Tissue Kit (Qiagen Inc.; Chatsworth CA). Two specific primers, TTATTGAGAATAGAGGACATCTG and ATGCTCAGAAAACTCCATAAC for the murine V_H^{62} were used to amplify by PCR a segment of 520

bp from genomic DNA. The PCR conditions were as follows: 45 sec at 94°C, 45 sec at 54°C and 45 sec at 72°C for 30 times. The PCR products were cloned in pGEM-T vector (Promega; Madison WI). Six clones from the genomic DNA of the spleen inoculated 17 days earlier and four clones from the genomic DNA of tranfectoma B cells (Sollazzo et al., *supra*, 1989) were sequenced on both strands by dideoxy termination method with Sequenase 2.0 DNA sequencing kit (USB; Cleveland OH) using two primers, AACAGTATTCTTTCTTTGCAGG and TTATTGAGAATAGAGGACATCTG, annealing 10 bp before the first codon of the FR1 and at the 3' end of the FR4, respectively.

The DNA of a chimeric immunoglobulin H chain gene consisting of a murine rearranged V_H region joined with a genomic human γ 1 C region was inoculated into the spleen of mice. This gene is under the control of tissue-specific regulatory elements (promoter and enhancer). The configuration of the plasmid DNA γ 1WT containing the H chain gene is shown in Figure 1. Mice were inoculated via the intrasplenic route once. A first pilot experiment compared the intraspleen route with other routes of inoculation, for example, intramuscular, subcutaneous, and intravenous, to verify the correctness of the hypothesis and to control for tissue-specificity. Table 1 shows the anti-immunoglobulin response determined by an ELISA method in mice inoculated through the various routes with the number of injections in each case. A marked antibody response was seen only in mice inoculated once via the intrasplenic route (group I). Mice inoculated once via the intrasplenic route and boosted intravenously three times (group V) also responded but because the three additional intravenous injections yielded a substantially similar antibody titer, a logical conclusion is that the antibody response seen in group V

reflects mainly the effect of intraspleen inoculation. The subcutaneous route yielded a weak response in two mice only (group III). No antibody response was detected in mice inoculated four times intramuscularly or intravenously (groups II and IV). Thus, the use of an immunoglobulin H chain gene under the control of tissue specific regulatory elements yielded immunity only after intraspleen inoculation.

Table 1. Production of Antibodies Reacting with the γ 1WT Protein in C57Bl/6 Mice Inoculated with γ 1WT DNA: Effect of the Route of Inoculation

Group	Route of Inoculation	Injections (no.)	Mice (no.)	Responders (no.)	Antibody titer ^a (log)
I	i.s.	1	4	4/4	3.1 \pm 0.4
II	i.m.	4	4	0/4	\leq 2.3 ^b
III	s.c	4	4	2/4	2.6
IV	i.v	4	4	0/4	\leq 2.3
V	i.s + i.v.	1+3	4	4/4	3.2 \pm 0.3

^a Values of antibody titer were measured and calculated on sera collected 21 days after the first inoculation.

^b The preinoculation value of a large pool of mice was 2.3 (log). The end-point positive serum dilution on which the titer was calculated was an OD value (A_{492}) \geq 0.200.

The kinetics of the antibody response was determined. In repeated experiments, intraspleen inoculation is reproducibly effective in initiating the immune response. The mean average antibody (log) titer ranged from 3.2 to 4.4, depending on the experiment (Table 2). The anti-immunoglobulin response was not merely due to a non-specific, polyclonal activation since mice injected with plasmid pSV2Neo, which lacks the

immunoglobulin coding region of plasmid DNA γ 1WT, failed to mount any detectable antibody response (Table 2). The time course of the anti-immunoglobulin response was further analyzed. Antibodies were detectable as early as day 5 with a peak on day 21 (Figure 2A). Thereafter, the antibody response dropped progressively but did not disappear. Circulating antibodies were still detectable 200 days after a single DNA inoculation.

Table 2. Production of Antibodies Reacting with the γ 1WT Protein in the Serum of C57B1/6 Mice After a Single Intraspleen Inoculation of DNA

Antibody titer (log)^a

Experiment number	Material inoculated	Mice (no.)	Responders (no.)	Mean \pm SD	Range
1	γ 1WT	14	14/14	3.6 \pm 0.2 ^b	3.2-3.8
2	γ 1WT	7	7/7	3.6 \pm 0.2	3.2-3.8
3	γ 1WT	9	9/9	4.1 \pm 0.2	3.8-4.4
4	pSB2neo	7	0/7	\leq 2.3	--
5	Saline	3	0/3	\leq 2.3	--

^a Values of antibody titers were measured and calculated on sera collected 21 days after the first inoculation.

^b The preinoculation value of each mouse was \sim 2.3(log). The end-point positive serum dilution on which the titer was calculated was an OD value (A492) \geq 0.200. Sera were tested in duplicate. The experiments and the ELISA were done independently and at different times.

The kinetics of transgene H chain immunoglobulin production were determined. Using an ELISA capture assay specific for the human $\gamma 1$ C region we detected the H chain transgene product in the serum of all mice inoculated with plasmid DNA $\gamma 1$ WT (Table 3). Mice injected with control plasmid pSV2Neo were consistently negative. Using a titration curve constructed with known amounts of human γ -globulins we estimated that at their highest detection value the concentration of the transgene product ranged between 7.3 and 32.1 ng/ml (minimal and maximal means of independent experiments) (Table 3). Values in individual mice varied between 1 and 72 ng/ml. A kinetic survey showed that the transgene H chain immunoglobulin product became detectable starting from day 10 (Figure 2b). Over numerous detections and several experiments we found that maximal detection, which varied from animal to animal, ranged between day 17 and 21. The H chain transgene product could not be detected beyond day 26 possibly due to the formation of immune complexes with anti-immunoglobulin antibodies. Thus, inoculation of an immunoglobulin H chain DNA via the intrasplenic route yielded a measurable secretion of the transgene immunoglobulin product in 100 percent of cases until the day 26.

Table 3. Detection of the Transgene Immunoglobulin Product in the Serum of C57B1/6 Mice After a Single Intraspleen Inoculation of DNA

				Production (ng/ml)	
Experi- ment number	Material inoculated	Mice (no.)	Producers (no.)	Mean \pm SD	Range
1	γ 1WT	14	14/14	7.3 \pm 7.6 ^a	1.0-21.1
2	γ 1WT	7	7/7	32.1 \pm 22.7	10.3-72
3	γ 1WT	9	9/9	9.3 \pm 5.1	5.1-15
4	pSV2neo	7	0/7	--	--
5	Saline	3	0/3	--	--

Values of transgene product in the serum represented correspond to the day of maximal detection for each individual mouse. Determination of circulating transgene immunoglobulins was done as detailed in Materials and Methods. The experiments and the ELISA were done independently and at different times.

The synthesis of transgene mRNA in the spleen of immunized mice was determined. *In vivo* transcription of the transgene was studied in spleen tissues of mice at various times after inoculation with plasmid DNA γ 1WT. The poly A(+) mRNA was prepared and reverse-transcribed, and the cDNA was amplified by polymerase chain reaction (PCR). Because the γ 1WT gene is a chimeric gene, we used primers specific for the human γ 1 C and the murine V_H regions, respectively. A 466 bp DNA segment from the human γ 1 C region and a 198 bp DNA segment from the murine V_H region were amplified independently (Figure 4A). mRNA was detected starting from day 3 until day 127. Whereas amplification of the human γ 1 C region in a naive mouse was negative, not surprisingly the murine V_H was

readily amplified as WT V_H^{62} (Figure 1) is a seemingly germline gene of the V_H^{7183} gene family (Figure 4A) (Sollazzo et al., *supra*, 1989). To verify the specificity, a Southern blot was performed on the DNA. As shown in Figure 4B, the amplified human $\gamma 1$ C segment co-migrated with the segment amplified from the pNeoy1 plasmid used as a positive control. To specifically probe for the V_H region, an experiment was performed in which the DNA used for inoculation was modified in the CDR3 to contain the coding sequence for three repeats of the tetrapeptide Asn-Ala-Asn-Pro ($\gamma 1$ NANP) as a nucleotide marker (Sollazzo et al., *supra*, 1990a) (Figure 1). The 240 bp DNA fragment amplified from cDNA gave a strong and specific hybridization band using a probe specific for the modified CDR3 segment (Figure 4C). No hybridization was observed in the amplified product from an unmanipulated spleen (Figure 4C). Taken together, these results demonstrate that following a single intraspleen inoculation, the H chain transgene is taken up by spleen lymphocytes and transcribed.

Somatic mutations were determined. DNA sequencing was used to determine whether persistence *in vivo* in the host cell DNA would cause the transgene to undergo somatic mutation. Because somatic mutation is property of the VDJ coding region (Griffiths et al., Nature 312:271-275 (1984)), this region only was characterized. The VDJ coding region (520 bp) was amplified from genomic DNA using specific primers as described above. Altogether, sequencing was done in six clones from genomic DNA of an inoculated spleen and four clones from genomic DNA of transfectoma B cells which served as reference. The nucleotide sequence of the six clones showed no mutation with the exception of a single conservative (C to T) mutation in framework 3 in clone

SP7. A single (C to T) mutation was also observed in framework 2 in clone TR38 from transfectoma B cells DNA (Figure 5). Thus, the VDJ coding region of the transgene retrieved in an integrated form 17 days after intraspleen inoculation did not show evidence of hypermutation. Thus, a lack of somatic mutation in the transgene *in vivo* was observed.

Anti-immunoglobulin antibodies were found to react with the V region of the H chain transgene. Polyclonal anti-immunoglobulin responses are predominantly directed against antigenic determinants of the C region. However, antibodies to V region determinants, for example, idiotypes and allotypes, are also usually produced. To determine whether the antibody response following inoculation of plasmid DNA $\gamma 1$ WT was also directed at V region determinants of the H chain transgene product, the sera of four were tested mice for binding to a panel of engineered antibodies to delineate specific reactivities. Figure 6A illustrates the composition of the H chain of the three immunoglobulin molecules used in the mapping experiment. $\gamma 1$ WT is the prototype immunoglobulin encoded by the $\gamma 1$ WT plasmid, as described in Figure 1; $\gamma 2$ bWT is an antibody possessing the WT V_H region joined to a murine $\gamma 2$ b C region; and $\gamma 2$ b V_H^{36-65} is an antibody possessing the murine V_H^{36-65} region joined to a murine $\gamma 2$ b C region. In all three instances, the L chain was $\gamma 1$. Serum anti-immunoglobulin antibodies from immunized mice bound in ELISA $\gamma 1$ WT and $\gamma 2$ bWT but not $\gamma 2$ b V_H^{36-65} (Figure 6B), suggesting that, following somatic transgene immunization, mice also produced antibodies against the V region of the H chain transgene product.

These results demonstrate that a nucleic acid molecule can be administered to a lymphoid tissue, the spleen, to elicit an immune response.

5

EXAMPLE II

In vivo Role of B Lymphocytes in Somatic Transgene Immunization

10

This example describes the role of B lymphocytes in somatic transgene immunization.

15

The preparation of plasmids and immunization are described below (Xiong et al., Proc. Natl. Acad. Sci. USA 94:6352-6357 (1997)).

20

Plasmid γ 1NANP (Sollazzo et al., *supra*, 1990a) carries a chimeric H chain gene in which a productively rearranged murine V region gene is joined to a human γ 1 C region gene. The V region of this H chain gene was modified in the third complementarity determining region (CDR3) by introduction of the nucleotide sequence coding for three Asn-Ala-Asn-Pro repeats (Sollazzo et al., *supra*, 1990a). The promoter and enhancer elements in this plasmid are those constitutively existing in Ig H chain genes and have been described previously (Sollazzo et al., *supra*, 1989). Plasmid pSVneo is the original plasmid vector that lacks the murine V region and the human γ 1 C region genes (Mulligan and Berg, *supra*, 1981).

30

The plasmid DNA was prepared from transformed DH5y *Escherichia coli* according to Example I. For DNA inoculation, eight to ten week old C57BL/6 (H-2b) female mice (Jackson Laboratories; Bar Harbor ME) were

inoculated with plasmid DNA (100 μ g in 30 μ l of sterile saline solution) directly into the spleen (see Example I).

Antibodies to γ 1NANP were detected on 96-well polyvinyl microtiter plates coated with affinity-purified antibody γ 1NANP (2.5 μ g/ml). Sera were diluted in PBSA. The bound antibodies were revealed using a HP-conjugated goat antibody to mouse γ -globulins absorbed with human γ -globulins (Pierce; St. Louis MO). The bound peroxidase was revealed by adding o-phenylenediamine dihydrochloride and H_2O_2 . Tests were done in duplicate.

The presence of transgene H chain immunoglobulins in the serum was detected using a capture ELISA (see Example I; Billetta and Zanetti, *supra*, 1992).

Ten milligrams of the tissue were digested in the presence of protease and the cell lysates were loaded onto the QIAamp spin column (Qiagen, Inc.; Chatsworth CA). After washing twice by centrifugation, the DNA was eluted from the column with distilled water and quantitated on a 1% agarose gel. PCR was performed with a total of four sets of primers, pCL and pCD; pSE and pNAD; pNEL and pNED; and p γ A1 and p γ A2. pCL (from -107nt to -85nt: 5'-TTATTGAGAATAGAGGACATCTG-3'; and pCD (from 459nt to 439nt: 5'-ATGCTCATAAACTCCATAAC-3'; were used to amplify the whole VDJ region of the transgene. pSE (from -32nt to -11nt: 5'-AACAGTATTCTTTCTTTGCAGC-3'; and pNAD (from 352nt to 333nt: 5'-GAGAGTAGGGTACTGGGTTT-3'; were specific for amplification of the genetic marker, (NANP)₃ in CDR3. pNEL (from 169nt to 189nt: 5'-AGCACCTACTATCCAGACACT-3'; and pNED (from 366nt to 346nt: 5'-GTAGTCCATACCATGAGAGTA-3'; were the inner primers for nested PCR. p γ A1 (from 184nt to 201nt: 5'-

TGGGCCGCCCTAGTCACC-3'; and pYA2 (from 427nt to 408nt: 5'-CGTTTGGCCTTAGGGTTCAG-3'; were designed to amplify the murine β -actin gene according to the sequence indicated in (Harris et al., Gene 112:265-266 (1992)). The PCR

5 consisted of 30 cycles at 94°C for 45 sec, 58°C for 45 sec, and 72°C for 45 sec; 0.3 μ M each primer; 0.2 mM each deoxynucleotide; 1.5 mM MgCl₂ in 20 mM Tris-HCl, pH 8.4 and 50 mM KCl; and 1 unit of Taq DNA polymerase (Gibco BRL; Gaithersburg MD). PCR products for Southern

10 blot analysis were resolved in 1% w/v agarose gel and blotted onto HYBOND-N nylon membrane (Amersham; Cleveland, OH). The membranes were hybridized with the oligonucleotide pNAD labeled using T4 polynucleotide kinase forward reaction in presence of (γ^{32} P-ATP).

15 For DNA sequencing, a 566 bp DNA fragment containing the whole VDJ coding region was amplified from splenic genomic DNA using two primers (pCL and pCD) specific for the rearranged murine V_H. This fragment was

20 subcloned into the pGEM-T vector (Promega; Madison WI). The plasmid DNA was extracted from transformed DH5 γ *Escherichia coli* and sequenced by dideoxy termination method with SEQUENASE 2.0 DNA Sequencing Kit (USB; Cleveland, OH) using two primers (pSE and pCD) annealing

25 in front of the FR1 and at the end of FR4 from opposite directions (see Figure (11) 7B).

30 For fluorescence-activated cell sorting (FACS), spleen cells were prepared by grinding the spleen tissue harvested 15, 21 and 28 days after inoculation, or from naive mice. The cell suspension was washed twice with 0.5% PBSA and the red blood cells were removed by treatment with lysing buffer (Sigma; St. Louis MO). The lymphocytes were differentially stained with

35 phycoerythrin (PE)-conjugated rat anti-mouse Ly-5 (B-220)

Pan B-cell (Caltag; San Francisco CA), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (Caltag) and FITC-conjugated rat anti-mouse CD8 (Caltag) for 20 min at 4°C. The cell suspension was washed twice in 0.5% PBSA and resuspended at the concentration of 5×10^6 cells/ml in DMEM (Irvine Scientific; Irvine CA). The cells were sorted on a FACSTAR (Becton & Dickinson; San Jose CA) at the Flow Cytometry core facility of UCSD Cancer Center. Genomic DNA was extracted from 1×10^6 B or T lymphocytes using the QIAAMP Blood kit (Qiagen). The DNA fragments were amplified by PCR and run on a 1% agarose gel. They were subsequently transferred to a nylon membrane for Southern blot hybridization using the (^{32}P)-labeled pNAD oligonucleotide.

The experiments resulted *in vivo* production of transgene H chain immunoglobulins and a humoral response. Plasmid DNA $\gamma 1\text{NANP}$ (Figure 1) was inoculated into the spleen of adult C57Bl/6 mice. Following a single intraspleen inoculation, transgene H chain immunoglobulins were detected (~ 12 ng/ml) in the serum of all mice inoculated with plasmid DNA $\gamma 1\text{NANP}$ but not in mice inoculated with the plasmid control pSVneo lacking the coding region for the H chain (Table 4). In all mice, antibodies against the $\gamma 1\text{NANP}$, protein were also detected starting from week 2 (Table 4). This is in agreement with the results described in Example I.

Table 4. Detection of transgene H chain immunoglobulins and antibodies to γ 1NANP after intraspleen inoculation of plasmid DNA.

Antibodies to γ 1NANP**

Inoculum (plasmid)	No. of mice	Transgene H-chain Ig* (ng/ml)	0 wk	2 wk	4 wk	6 wk
γ 1NANP	10	11.9	≤ 2.3	2.8 ± 0.3	2.9 ± 0.4	3.0 ± 3.0
pSVneo	4	0	≤ 2.3	≤ 2.3	≤ 2.3	2.4 ± 0.2

* Presence of transgene H chain immunoglobulins was determined at 2 wk after a single DNA inoculation. Detection was performed using a capture ELISA.

** Antibodies are expressed as a Log titer. Titer determined as the last serum dilution giving an OD reading >0.200 (A_{492}).

Kinetics and tissue distribution of the transgene *in vivo* were determined. To monitor the kinetics of detection of the transgene *in vivo*, genomic DNA extracted from the spleen of inoculated mice was analyzed by PCR and Southern blot hybridization at various times after inoculation. As shown in Figure 7 (A, left panels), amplification of the transgene VDJ region was visible up to 12 weeks after a single DNA inoculation. No amplification was seen in the subsequent time points (16, 24, 36 and 52 weeks). To control for specificity and increase the sensitivity of the reaction, two additional PCR assays were performed using primers designed to anneal to sites within the VDJ region. One set of primers (pSE/pNAD) specifically amplified the (Asn-Ala-Asn-Pro)₃ coding sequence, and another (inner primers: pNEL/pNED) served for nested PCR (see Figure 7B). The results confirmed those obtained with VDJ

amplification. Southern blot analysis using a probe specific for the NANP-coding region further confirmed the PCR results (Figure 7A, right panels). Thus, the transgene H chain persisted *in vivo* for a period of 3 months. To determine tissue distribution of the transgene *in vivo*, genomic DNA was extracted from various lymphoid, for example, spleen, lymph nodes and bone-marrow, and non-lymphoid, for example, liver, kidney, lung and muscle, tissues explanted at different times, and analyzed for specific amplification of the transgene VDJ by PCR. Whereas an amplification product was readily visible in splenic genomic DNA, no specific amplification occurred in any of the other tissues. This did not vary at any of the time points analyzed (Figure 7C, left panels). Southern blot analysis confirmed the PCR results (Figure 7C, right panels).

To formally demonstrate that B lymphocytes were in fact the target cell population *in vivo* for the transgene, the following experiment was performed to detect the transgene in splenic lymphocytes. Starting from the second week after plasmid DNA inoculation, splenic B and T lymphocytes were isolated to a high degree of purity (97-99%) by FACS sorting (Figure 8). The genomic DNA was extracted from the two cell populations and amplified by PCR using the same sets of primers as in Figure 7. At the 15 day time point, distinct amplification products were readily detectable in both B and T lymphocytes (Figure 8, left panel). However, at both the 21 and 28 day time points, specific amplification was observed only in B cells (Figure 8, middle and right panels). Southern blot hybridization confirmed the specificity of the amplification products. These results suggested that B lymphocytes in the spleen are the target cell population in which the transgene

persists for a long time. Together with the fact that the transgene could not be amplified from peripheral blood lymphocytes, these results indicate that the likely destiny of spleen B lymphocytes harboring the transgene is to remain localized in the tissue in which they were transfected.

The transgene was sequenced from genomic DNA. The immunogenic potential of a transgene-encoded product depends on the fact that no sense somatic mutation will affect the nucleotide sequence of the transgene while harbored *in vivo*. Hypermutation is of frequent occurrence in the VDJ region of Ig, and in particular in the CDRs, a fact in agreement with the notion that hypermutation takes place during antigen selection and affinity maturation of the antibody response (Griffiths et al., *supra*, 1984). Although the transgene used in this study lacks a transmembrane domain, rendering cell surface anchoring unlikely, experiments were nevertheless performed to assess the accumulation of mutations as a result of protracted *in vivo* persistence in integrated form. The transgene VDJ region was amplified from splenic genomic DNA, subcloned and sequenced by the dideoxy termination method. No evidence of hypermutation was found in the VDJ region of the transgene even after 3 months *in vivo* (Table 5).

Table 5. Lack of transgene mutations in PCR-generated clones from splenic genomic DNA.

Time (wk)	No. of clones sequenced	No. of clones mutated	No. of nucleotides mutated	Rate of mutation* (%)
2	6	1/6	1**	2.9×10^{-4}
4	3	0/3	0	
12	3	0/3	0	

* Number of mutations per total number of base pairs sequences.

** A silent (C to T) mutation in FR3.

These results demonstrate that *in vivo* inoculation with plasmid DNA resulted in expression of the transgene in B cells of the spleen for at least three months.

EXAMPLE III

Immunity to *Plasmodium falciparum* Malaria Sporozoites by Somatic Transgene Immunization

This example describes administration of a nucleic acid molecule encoding a B-cell epitope of *P. falciparum* malaria parasite to induce an immune response against the parasite antigen.

The protocols used are described below (Gerloni et al., Nature Biotech. 15:876-881 (1997)).

Eight to ten week old C57BL/6 (H-2^b) mice were purchased from the Jackson Laboratories (Bar Harbor ME). Mice were maintained in the animal facility of the

University of California, San Diego, and were handled according to UCSD and NIH regulations.

5 γ 1NANP is described in Example I. pSV2Neo is the original plasmid forming the backbone of the pNyl vector without the human γ 1 C region gene. This plasmid was used as a control in the immunization experiments. Plasmid DNA was prepared from DH5 α *Escherichia coli* and purified according to standard procedures.

10 The antigenized antibody γ 1NANP was produced and purified as described previously (Billetta et al., *supra*, 1991) and stored at -20°C until use. The recombinant antigen R32LR consisting of 30 repeat of the tetrapeptide NANP fused to 32 amino acids derived from 15 the tet^r region of the PAS1 plasmid was prepared as described (Wirtz et al., Exp. Parasitol. 63:166-172 (1987)). A synthetic peptide containing multiple repeats of the NANP sequence, (NANP)_n. Control peptide 20 DENGNYPLQC is from the sequence of the human invariant chain. *P. falciparum* sporozoites were produced in *Anopheles freeborni* mosquitos infected as described (Wirtz et al., *supra*, 1987).

25 The detection of antibodies to synthetic peptide (NANP)_n was done as described in Example II. Antibodies to intact γ 1NANP protein and R32LR antigen were detected on plates coated (2.5 μ g/ml) with the corresponding substrate by drying at 37°C. After 30 coating, wells were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) 0.15 M, pH 7.3. Wells were incubated overnight at 4°C with mice sera diluted in 1% BSA-PBS, containing 1% TWEEN-20 (PBSA). The bound antibodies were revealed using a goat antibody 35 to mouse immunoglobulin absorbed with human

immunoglobulin and conjugated with horseradish peroxidase (HP) (Sigma; St. Louis MO). The bound peroxidase activity was revealed by adding o-phenylenediamine dihydrochloride and H₂O₂. Plates were read after 30 min in a micro-plate reader (VMAX; Molecular Devices) at 492 nm. Control plates coated (2.5 µg/ml) with synthetic peptide DENGNYPLQC were coated similarly to plates coated with the synthetic peptide NANP.

The presence of the transgene H chain immunoglobulins in the serum of mice was detected using a capture ELISA assay (Billetta and Zanetti, *supra*, 1992) as in Example I. Ig class determination of anti-(NANP)_n and anti-γ1NANP antibodies was done by ELISA using isotype-specific rabbit antibodies (Mouse Typer Sub-isotyping kit; Bio-Rad; Hercules CA). Microtiter wells were coated with synthetic peptide (NANP)_n (2.5 µg/ml) at 4°C in 0.1 M carbonate buffer, pH 8.6, or with γ1NANP protein (2.5 µg/ml) in 0.9% NaCl by drying at 37°C. Plates were blocked with 1% BSA. Pooled mouse sera diluted in PBSA were incubated overnight at 4°C. After washing, 100 µl of anti-isotype antibodies at the dilution recommended by the manufacturer were incubated for 3 hrs at room temperature. The bound antibodies were revealed by adding (100 µl/well) of HP-conjugated goat antibody to rabbit Ig diluted 1:3,000 in PBSA for one hr at room temperature. The test was continued as described above. Tests were done in duplicate.

Sera diluted 1:50 were assayed for immunofluorescence reactivity with air dried *P. falciparum* sporozoites at various dilutions (1:25 to 1:800). The assays were performed as previously described (Wirtz et al., *supra*, 1987). Fluorescence intensity was graded from 0 to 4+, with 0 indicating no fluorescence

detectable and 4+ indicating intense fluorescence over the entire surface of the sporozoites. Sample with \geq fluorescence intensity were considered positive.

5 Mice were inoculated with 100 μ g of plasmid DNA
in 30 μ l of sterile saline solution intraspleen as
detailed under Example I. In the experiment described in
Table 6 mice, were boosted with 100 μ g of plasmid DNA
10 γ 1NANP in saline administered intravenously via the tail
vein. Inoculation of DNA via other routes was performed
as described (see Example I).

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Table 6. Titers (\log_{10}) of antibodies reacting with NANP peptide after priming and booster immunizations.

Group	Priming*	Booster	No. of mice	Primary immune response (days)	Secondary immune response (days)
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I	γ 1NANP DNA	γ 1NANP DNA	4	≤ 2.3	2.6	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0	2.9 ± 0	2.9 ± 0
II	γ 1NANP DNA	γ 1NANP protein	4	≤ 2.3	2.6	2.9	2.8 ± 0.2	3 ± 0.2	3.6 ± 0.3	3.7 ± 0.4
III	psvneo DNA	γ 1NANP protein	4	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3
IV	γ 1NANP protein	γ 1NANP protein	4	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	2.4 ± 0.3	2.5 ± 0.4	2.6 ± 0.6
V	OVA protein	OVA protein	4	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3	≤ 2.3

* All priming injections were done through the intraspleen route. Booster injections were done on day 200. In all but one group (group 1, which was done intravenously) booster injections were done subcutaneously.

Mice were inoculated *i.s.* with affinity-purified γ 1NANP protein in sterile saline solution. The surgical procedures were as described above. Mice were immunized with affinity-purified γ 1NANP protein emulsified in complete Freund's adjuvant (50 μ g per mouse) subcutaneously. Mice that were boosted with the γ 1NANP protein received 50 μ g of the protein emulsified in incomplete Freund's adjuvant subcutaneously or 50 μ g of the protein adsorbed on alum intraperitoneally. 10^5 irradiated sporozoites in incomplete DMEM were injected intraperitoneally in a 0.4 ml volume. Mice were bled via the retro-orbital route.

Inoculation of plasmid γ 1NANP DNA γ 1NANP induces a primary response against the peptide NANP. Previously, immunization was demonstrated with an antibody antigenized with three NANP repeats in the CDR3 of the heavy (H) chain (γ 1NANP) induced antibodies that recognized the CS repeat epitope in mice and rabbits (Billetta et al., *supra*, 1991). These antibodies also reacted with *P. falciparum* sporozoites by indirect immunofluorescence. To determine whether a similar antibody response against (NANP)₃ peptide could be induced, immunocompetent C57/Bl6 mice were inoculated with plasmid DNA coding for the γ 1NANP gene (Figure 9). These experiments were based on the work showing that an immunoglobulin H chain transgene could induce immunity if inoculated directly into the spleen (*i.s.*) (see Example I) but not if introduced using other routes of immunization (intramuscular, intravenous, intradermal and subcutaneous)

Table 6 summarizes the ELISA antibody responses in which anti-NANP peptide antibodies were found in mice primed with the H chain transgene (γ 1NANP DNA) (groups I

and II). Antibodies appeared by day 14 and reached a plateau by day 28 (log 2.8) (Table 6). Circulating antibodies persisted through day 200 when mice received a booster injection. The antibody response against the intact antigenized antibody γ 1NANP paralleled the response against the synthetic peptide. Mice inoculated intrasplenically with 50 μ g of the γ 1NANP protein (group IV) failed to mount any measurable anti-peptide response, although a modest elevation in titer against the intact γ 1NANP antibody was measured. Control groups injected with either the pSVneo plasmid or with ovalbumin failed to develop any antibody response above background titers higher than the pre-immunization values. No binding was observed when the same sera were tested on the synthetic peptide DENGNYPLQC used as a control.

Memory response against the NANP peptide was induced by γ 1NANP DNA. A single intrasplenic inoculation of plasmid γ 1NANP DNA γ 1NANP was sufficient to induce immunologic memory against the (NANP)₃ peptide expressed in the CDR3 of the H chain transgene. Table 6 shows the secondary anti-peptide response following a subcutaneous booster injection of the γ 1NANP protein in incomplete Freund's adjuvant (groups II and IV). The antibody titer against the synthetic NANP peptide rose in all animals in group II, and paralleled the response against the intact γ 1NANP protein. In contrast, no anamnestic response occurred in mice boosted with a second intravenous injection of γ 1NANP DNA (group I) perhaps because of the rapid degradation of plasmid DNA by plasma DNases. The antibody response in mice primed by *i.s.* inoculation with soluble γ 1NANP protein and boosted with γ 1NANP protein subcutaneously (group IV) was similar to that seen with primary immunizations using the recombinant protein

alone. No antibody responses against NANP were detected in control mice (groups III and V).

Transgenic H chain immunoglobulins were detected in the serum of all mice inoculated with plasmid DNA γ 1NANP DNA (Figure 15D) with a concentration ranging between 4.8 and 30.1 ng/ml (average 11.9 ± 6.2 ng/ml).

Immunization with γ 1NANP DNA induced immunologic memory response against *P. falciparum* sporozoites. To verify whether somatic transgene immunization could prime for immunologic memory upon encounter with the native CS protein of the parasite, mice were boosted by a single injection of *P. falciparum* sporozoites. The resulting antibody response was measured by ELISA. For comparison, mice were divided into two groups. One group was primed *i.s.* with plasmid DNA γ 1NANP (or its control γ 1WT). A second group was primed subcutaneously with antigenized antibody γ 1NANP in complete Freund's adjuvant. Forty-five days after priming, mice were boosted with a single intraperitoneal injection of 10^5 *P. falciparum* sporozoites or with antigenized antibody γ 1NANP in incomplete Freund's adjuvant by subcutaneous injections. Control groups included mice primed with plasmid γ 1WT DNA or saline, and subsequently boosted with sporozoites. Mice primed with γ 1NANP DNA and boosted with sporozoites (Figure 10) mounted a secondary response against NANP that was absent in mice primed with control plasmid DNA or with saline alone. Moreover, the anamnestic responses to sporozoites were greater in mice primed with γ 1NANP DNA than in mice primed with the antigenized antibody γ 1NANP in complete Freund's adjuvant (CFA) (Figure 10A and 10C). Similar results were obtained when the sera were tested by ELISA

on recombinant R32LR as capture antigen (Figure 10B and 10D).

Figure 11 further demonstrates that γ 1NANP DNA primed antibody responses to the native protein as found on the sporozoite surface. Anti-CS repeat antibodies were boosted in mice primed with γ 1NANP DNA (Figure 11A), that received sporozoites on day 45. Antibody responses against the NANP repeat were substantially higher than those against the γ 1NANP protein. Similarly, mice primed with antigenized antibody γ 1NANP (Figure 11B) also responded to the booster injection with sporozoites, although less dramatically than did the mice primed with the transgene vaccine. These results indicate that STI primed for immunologic memory and resulted in a strong secondary anti-sporozoite response.

These sera also reacted strongly with the surface of air-dried sporozoites by indirect immunofluoresence assay (Table 7), confirming that the DNA-immunized mice had been primed with a B cell epitope with a conformation that was substantially similar to that present on the surface of the target pathogen.

Table 7. Antibodies reacting with *Plasmodium falciparum* sporozoites by IFA.

Priming*	Booster*	IFA reactivity
		Titer ^y
γ1NANP DNA	--	25
γ1NANP DNA	Sporozoite	400
γ1NANP DNA	γ1NANP protein	50
γ1NANP protein	--	0
γ1NANP protein	Sporozoite	50
γ1NANP protein	γ1NANP protein	800

*Priming and booster injections refer to Figure 4. Sera were tested as pools of four mice each. Values shown represent the reciprocal of the last positive dilution.

Analysis of antibody isotype responses after priming showed that mice inoculated *i.s.* with plasmid γ1NANP DNA produced IgM and, to a lesser extent, IgG2a antibodies (Figure 12). Booster with *P. falciparum* sporozoites enhanced specific antibodies of all isotypes, in particular IgG1 antibodies. Similarly, booster with the γ1NANP protein promoted the formation of IgG1 antibodies and a minor but significant rise in IgG2a and IgM antibodies. By comparison, booster with sporozoites yielded proportionally higher isotype responses in mice primed with the γ1NANP DNA than in mice primed with the γ1NANP protein in complete Freund's adjuvant.

These results demonstrate that immunity to *P. falciparum* can be induced by administration of a nucleic acid molecule encoding a *P. falciparum* epitope.

EXAMPLE IV

Engineering Vaccines with Heterologous B and T Cell
Epitopes Using Immunoglobulin Genes

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This example describes the insertion of heterologous B and T cell epitopes into the CDRs of an immunoglobulin to enhance the immunologic response when administered as plasmid DNA.

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The experimental procedures are described below (Xiong et al., Nature Biotechnology, 15:882-886 (1997)).

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Plasmid $\gamma 1\text{NV}^2\text{NA}^3$ was engineered as described below. The EcoRI fragment of the productively rearranged murine VH (2.3 Kb) was cloned in vector pBluescript II KS to yield plasmid pVH. Site-directed mutagenesis was performed using two 21mer oligonucleotide primers, one (5'-CAAGAAAGG**TACC**TACTCTC-3') annealing in CDR3 to introduce 3bp (TAC, in bold) for the creation of an Asp718 site, and another (5'-AGTAATGG**CCA**TGGTAGCACC-3') annealing in CDR2 to introduce 3bp (CCA, in bold) for the creation of a NcoI site. These primers were annealed to the uracylated, complementary strand of pVH and the mutant strands were synthesized and ligated in the presence of T4 DNA polymerase and ligase. Plasmid pVH-TAC/CCA, containing two unique sites, one in CDR3 (Asp718) and the other in CDR2 (NcoI), was obtained after transformation, screening of individual colonies and confirmation by DNA sequencing (SEQUENASE 2.0 DNA Sequencing Kit; USB; Cleveland OH). A pair of complementary oligonucleotides, 5'-GTACCCAATGCAAACCCAAATGCAAACCCAAATGCAAACCCA-3' (sense) and 5'-GTACTGGGTTTGCATTTGGGTTTGCATTTGGGTTTGCATTGG-3' (antisense) coding for the (NANP)₃ sequence was

synthesized, annealed and cloned in the Asp718 site. A pair of complementary oligonucleotides 5'-CATGGTAATGCAAACCCAAATGTAGATCCCAATGCCAACCCA-3' (sense) and 5'-CATGTGGGTTGGCATTGGGATCTACATTTGGGTTTGCATTAC-3' (antisense) coding for the NANPNVDPNANP sequence was

similarly cloned into the NcoI site. The insertions and the proper orientation were verified by dideoxy sequencing (SEQUENCASE 2.0 DNA Sequencing Kit; USB). The 2.3Kb EcoRI fragment carrying the engineered CDR3 and CDR2 was then subcloned in the expression vector pNyl1 (Sollazzo et al., *supra*, 1989) upstream from the human γ 1 constant (C) region using the unique EcoRI site to yield plasmid γ 1NV²NA³. Plasmid γ 1NANP carries a productively-rearranged murine V region gene in which only the CDR3 was modified by introducing the nucleotide sequence coding for three NANP repeats (Sollazzo et al., *supra*, 1990a). The promoter and enhancer elements in these plasmids are those constitutively existing in Ig H chain genes (Sollazzo et al., *supra*, 1989).

Plasmid DNA was prepared from transformed DH5 α *Escherichia coli* according to procedures described in Example I. Eight week old C57Bl/6 (H-2^b) female mice were purchased from the Jackson Laboratories (Bar Harbor ME). Mice were maintained in the animal facility of the University of California, San Diego, throughout the duration of the experiments. Mice were inoculated with 100 μ g of plasmid DNA in 50 μ l volume of sterile saline solution as in Example I.

The recombinant antibodies γ 1WT and γ 1NANP were produced and purified as described previously (Billetta and Zanetti, *supra*, 1992; Sollazzo et al., *supra*, 1989) and stored at -20°C until use. The synthetic peptide containing multiple repeats of the NANP sequence was the

kind gift of Dr. A. Verdini (Monterotondo, Italy) and peptide NANPNVDPNANP was synthesized in the peptide synthesis facility of the University of California, San Diego.

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Antibodies to the (NANP)₃ and NANPNVDPNANP peptides were detected by ELISA on 96-well polyvinyl microtiter plate (Dynatech, Gentilly, VA) coated with synthetic peptide (2.5 µg/ml) in 0.1 M carbonate buffer, pH 8.6, by incubation overnight at 4°C. After coating, wells were blocked with 1% BSA in PBS and then incubated overnight at 4°C with mice sera diluted in phosphate buffered saline (PBS), 0.15 M, pH 7.3, containing in 1% bovine serum albumin (BSA) and 0.05 % TWEEN-20. The bound antibodies were revealed using a HP-conjugated goat antibody to mouse Ig absorbed with human Ig (Sigma) (1:10,000 dilution). The bound peroxidase was revealed by adding o-phenylenediamine dihydrochloride and H₂O₂. Plates were read after 30 min in a micro-plate reader (VMAX; Molecular Devices) at 492 nm.

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Detection of antigenized antibodies in culture supernatants and transgene H chain Ig in mice sera was performed by a capture ELISA assay (Billettea and Zanetti, *supra*, 1992). Briefly, culture supernatants or individual mouse sera (1:10 dilution) in PBS-BSA containing 0.05% TWEEN-20, were incubated overnight at 4°C on 96-well plates coated with goat antibody to human IgG1 (10 µg/ml). The concentration of the transgene H chain Ig was calculated by plotting O.D. values of test samples against a standard curve constructed with known amount of human IgG1 spiked into PBS-BSA 0.05% TWEEN-20 containing 10% normal mouse serum. The bound antibodies were revealed using a HP-conjugated goat antibody to human Ig (Sigma) (1:1,000 dilution). The assay was

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continued as described above. Tests were done in duplicate.

Detection of κ and λ light chains in circulating transgene H chain Ig was done with a modification of the above assay. Briefly, serum transgene H chain Ig were captured on 96-well plates coated with goat antibody to human IgG1 (10 μ g/ml) by incubation overnight at 4°C. The presence of murine light chains was assessed using a 1:2000 dilution of HP-conjugated goat antibodies to murine κ or λ light chains adsorbed with human Ig (Caltag; San Francisco CA). The assay was continued as described above. Tests were done in duplicate.

The engineering of two distinct epitopes in the same Ig V region gene was performed in the CDR3 and the CDR2 which contain a Asp718 (Sollazzo et al., *supra*, 1990b) and NcoI site, respectively. In the expressed proteins, both CDRs are loops interconnecting β -strands on the same β -sheet of the V domain. A modification of these two CDRs was expected to be compatible with proper VH/VL scaffolding, whereas engineering of the CDR1, which connects two different sheets of the V domain, could result in misfolding of the polypeptide. The B cell epitope used consisted of three repeats of the tetrapeptide Asn-Ala-Asn-Pro (NANP) from the CS antigen of *P. falciparum* parasite (Zavala et al., *supra*, 1985).

The Th cell epitope used is the peptide Asn-Ala-Asn-Pro-Asn-Val-Asp-Pro-Asn-Ala-Asn-Pro (NANPNVDPNANP), a conserved peptide sequence located in the 5' region of the CS antigen of *P. falciparum*. This peptide is recognized by immune human CD4⁺ T lymphocytes

(Nardin, et al., Science 246:1603-1606 (1989), is immunogenic for several MHC haplotypes in the mouse (Munesinghe et al., *supra*, 1991) and has been included in multiple-antigen-peptide vaccines for malaria.

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The CDR3 and CDR2 of pVH were engineered as illustrated in Figure 15. The 2.3 Kb EcoRI DNA fragment carrying a productively-rearranged murine V_H cloned into pBluescript(pVH) was modified by oligonucleotide site-directed mutagenesis to introduce two unique cloning sites, Asp 718 site in CDR3 (Sollazzo et al., *supra*, 1990a) and NcoI in CDR2 (pVH-TAC/CCA). A pair of complementary synthetic oligonucleotides coding for three NANP repeats was cloned into the Asp 718 site whereas the pair coding for the NANPNVDPNANP sequence was cloned into the NcoI site of pVH-TAC/CCA. Nucleotide insertion and the correct orientation were checked by PCR and confirmed by sequencing (Figure 15A). The engineered 2.3 Kb EcoRI fragment was then cloned into the unique EcoRI site of the expression vector pNyl1 to yield plasmid γ 1NV²NA³ (Figure 15B). The V region gene of plasmid γ 1NV²NA³ codes, therefore, for two distinct epitopes of the CS antigen, one in CDR3 and the other in CDR2.

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In vivo expression of transgene H chain antibodies was determined. As described in Example I, following intraspleen inoculation of plasmid DNA coding an Ig H chain gene, transgenic Ig were invariably detected in the circulation in amounts ranging between 15 and 30 ng/ml 10. Similar amounts were detected in mice inoculated with the antigenized H chain gene coding for the NANP epitope in CDR3 (see Example III). Mice inoculated with plasmid γ 1NV²NA³ secreted transgene H chain Ig in amounts comparable to those secreted by mice inoculated with plasmid DNA γ 1NANP (29.4 vs. 33.3 ng/ml)

(Figure 16A). These results indicate that, as observed *in vitro*, the modifications in the two CDR loops did not impact folding and secretion of transgene H chain Ig associated with endogenous γ light chains (Figure 16B versus 16C). Collectively, these results suggest that transgene H chains with insertion of heterologous dodecapeptides in two CDRs are handled *in vivo* as conventional Ig H chain genes and pass quality control mechanisms in the endoplasmic reticulum similarly to endogenous Ig molecules.

To determine *in vivo* immunogenicity, immunogenicity of secreted transgene H chain Ig carrying the two heterologous epitopes was analyzed by direct intraspleen inoculation of plasmid γ 1NV²NA³ and by comparing the antibody response in these mice to that of mice inoculated with plasmid γ 1NANP. All mice mounted a humoral antibody response to the human constant region of the transgene product proving that immunization took place. Mice of both groups produced anti-(NANP)₃ antibodies, indicating that in both instances, the CDR3 loops were immunogenic (Figure 17). However, the anti-NANP response in mice inoculated with plasmid γ 1NV²NA³ was higher than in mice inoculated with plasmid γ 1NANP (Figure 17A versus 17B). Interestingly, whereas mice inoculated with plasmid γ 1NV²NA³ produced antibodies reactive against both (NANP)₃ and NANPNVDPNANP peptides (Figure 17B and 17D), mice inoculated with plasmid γ 1NANP produced antibodies against (NANP)₃ only (Figure 17A and 17C). Because antibodies to (NANP)₃ do not cross-react with NANPNVDPNANP, mice inoculated with plasmid γ 1NV²NA³ produced two distinct populations of antibodies, one against the (NANP)₃ peptide in CDR3 and the other against the NANPNVDPNANP peptide in CDR2.

These results demonstrate that the two engineered CDRs were independently immunogenic *in vivo* and that the presence of the Th cell determinant in CDR2 enhanced the production of antibodies against the B cell epitope in CDR3.

EXAMPLE V

Durable Immunity and Immunologic Memory to a Parasite Antigen Induced by Somatic Transgene Immunization

This example shows that inoculation of plasmid DNA carrying an immunoglobulin heavy chain gene under the control of tissue-specific regulatory elements leads to immunity and persistent immunologic memory against a peptide epitope.

The protocols used are described below (Gerloni et al., Vaccine 16:293-297 (1998)).

Eight week old C57Bl/6 (H-2^b) female mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained in the animal facility of the University of California, San Diego, throughout the duration of the experiments. Plasmid γ NANP (Sollazzo et al., *supra*, 1990a) and the γ 1NANP protein were prepared as in Example I and III.

Mice were inoculated with 100 μ g of plasmid DNA per injection. Five basic routes of inoculation were used. These are described under Example I.

Mice were immunized with affinity-purified γ 1NANP protein (50 μ g per mouse) either emulsified in complete Freund's adjuvant (CFA), subcutaneously, or

adsorbed onto alum, intraperitoneally. The booster immunizations consisted of a single injection of γ 1NANP protein (50 μ g/mouse) emulsified in incomplete Freund's adjuvant (IFA), subcutaneously. The times of booster
 5 are as indicated in the text and in the legend to Figure 14. Antibodies to intact γ 1NANP protein (NANP)_n were detected by ELISA as in Example II.

The immunity induced via STI was compared to
 10 conventional immunity using immunologic adjuvants. Immunoglobulins possess unique antigenic determinants clustered primarily in or around the CDRs (Rudikoff, Cont. Top. Mol. Immunol. 9:169-208 (1983); Billetta and Zanetti, Int. Rev. Immunol. 10:251-263 (1993)). The
 15 results represented in Figure 9 show the development of the primary antibody response directed against the (NANP)₃ amino acid sequence expressed in the CDR3 of the transgene H chain. Anti-NANP antibodies became detectable by the second week and increased through week
 20 6. No binding to the (NANP)_n peptide was detected in mice inoculated with plasmid control pSVneo. The anti-NANP antibody response induced through STI was compared to that elicited by immunization with the γ 1NANP protein administered in immunologic adjuvants. As shown in
 25 Figure 13, at week 6, the antibody titer measured in mice inoculated with DNA was higher than that in mice immunized with γ 1NANP protein in CFA or in alum, respectively.

STI induced long-lasting immunologic memory.
 Immunologic memory was tested by investigating the ability of primed mice to respond to a single booster dose of γ 1NANP protein in IFA given subcutaneously 6, 30
 or 104 weeks after the DNA inoculum. The anamnestic
 35 response against the NANP epitope was measured 2 and 5

weeks after booster. As shown in Figure 14, a specific anamnestic response occurred at all three time points after priming. NANP-reactive antibodies are still detectable after two years from priming. The booster effect was weaker in mice boosted at 6 weeks than in mice boosted at later times. In mice boosted on week 30, circulating antibodies persisted at a high titer (Log_{10} 3.5) for at least an additional 17 months. The booster response was specific since mice inoculated at the time of priming with plasmid DNA pSVneo had a much lower antibody titer. Booster with plasmid DNA intravenously did not yield any memory response.

These results demonstrate administration of a nucleic acid molecule results in persistent immunologic memory up to 2 years.

EXAMPLE VI

DNA Immunization in *relB*-deficient Mice Discloses a Role for Dendritic Cells in IgM to IgG1 Switch *in vivo*

This example describes the role of dendritic cells in the IgM to IgG switch *in vivo*.

The protocols are described below (Gerloni et al., Eur. J. Immunol. 28:516-524 (1998)).

Eight to ten week old C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor ME) and were kept in the animal facility of the University of California, San Diego. The generation of *relB* (-/-) and hemizygous (-/+) mice was described previously (Lo et al., *supra*, 1992). Bone marrow chimeras were constructed using as recipients irradiated (1100R) C57Bl/6J injected

with 7×10^6 bone marrow cells from *relB* (-/-) or (-/+) mice (Burkly et al. *supra*, 1995). Inoculation with plasmid DNA was performed eight weeks after bone marrow transfer.

5 γ 1NANP is described in Example I. In plasmid DNA γ 1NANP/GM-CSF, the murine GM-CSF coding sequence from plasmid p3159 was cloned at the 3' end of the CH3 domain through a Gly-Gly linker (Tao et al., *supra*, 1993). In plasmid DNA γ 1NANP/IL-2, the murine IL-2 coding sequence
10 from plasmid p3163 was similarly cloned at the 3' end of the CH3 domain. Plasmids DNA were prepared from DH5 γ *Escherichia coli* according to procedures (Sambrook et al., *supra*, 1989) as detailed in Example I.

15 The recombinant antibody γ 1NANP was produced and purified as described previously (Sollazzo et al., *supra*, 1989); Billetta and Zanetti, *supra*, 1992).

20 Mice were inoculated intraspleen with 100 μ g of plasmid DNA in 30 μ l of sterile saline solution as described in Example I. Booster injections (C57Bl/6 mice only) were administered on day 35 by a single subcutaneous injection (50 μ g per mouse) of affinity-purified γ 1NANP protein emulsified in incomplete Freund's
25 adjuvant.

30 Antibodies to the intact γ 1NANP protein were detected by enzyme-linked immunosorbent assay (ELISA) as in Example II. The isotype of antibodies was determined using rabbit antibodies specific for the various murine Ig classes (Mouse Typer Sub-isotyping kit; Bio-Rad; Hercules, CA) as in Example III.

35 A GM-CSF chimeric gene induced isotype switch. STI was induced using plasmid DNA γ 1NANP coding for an Ig

H chain gene in which the 3' end of the CH3 domain of the human constant region gene was chimerized in one case with murine GM-CSF and in another case with IL-2 cDNA (Figure 19). GM-CSF has been shown to augment the immunogenicity of tumor cell (Dranoff et al., Proc. Natl. Acad. Sci. USA 90:3539-3543 (1993); Levitsky et al. J. Immunol. 156:3858-3865 (1996)) and DNA (Xiang and Ertl, *supra*, 1995) vaccines possibly through its action on DC (Inaba et al., J. Exp. Med. 176:1693-1702 (1992)). It has been reported that immunization with tumor-derived idiotypic/GM-CSF fusion protein induces a potent anti-idiotypic response (Tao et al. *supra*, 1993), and inoculation with an idiotypic/GM-CSF chimeric gene protects against B-cell lymphoma in mice (Syrengeles et al., Nat. Med. 2:1038-1041 (1996)). IL-2 is required for growth of T cells and is a cofactor in activating B cells to secrete Ig (Smith, Science 240:1169-1176 (1988)).

In three independent experiments, inoculation with DNA/GM-CSF, but not DNA/IL-2, yielded a primary antibody response against TgIg of slightly greater magnitude than in mice inoculated with γ 1NANP DNA (Figure 20A). Mice inoculated with DNA/GM-CSF produced IgG1 antibodies against TgIg readily after priming (Figure 20B). As described in Example I, booster injection with antigenized antibody in Freund's adjuvant induced an IgG1 response, indicating that intraspleen inoculation of DNA does not hamper the class switch machinery. Thus, the effect observed with inoculation of DNA/GM-CSF can be attributed to a function mediated by GM-CSF. GM-CSF did not have any detectable effect on IgG2a antibodies but did modify the IgG2b response after booster immunization (Figure 21) in that it prevented the elevation of antibodies of this isotype upon booster. Since a similar effect was noted in mice receiving the DNA/IL-2, it is

possible that down-regulation of IgG2b antibodies is not specific for GM-CSF. Mice inoculated with DNA/GM-CSF produced the highest titer of IgG1 antibodies (Figure 21). These results indicate that inoculation of plasmid DNA containing an Ig H chain gene chimerized with the GM-CSF gene promoted IgM->IgG1 switch *in vivo*.

A lack of isotype switch was observed in *relB* (-/-) mice. Failure to produce IgG1 following inoculation with γ 1NANP DNA indicated that neither B cells nor DC presented TgIg peptides to T cells in immunogenic form sufficient to promote secretion of IL-4 and other cytokines required to drive switch to IgG1. Resting B cells are unable to activate naive T cells *in vivo* due to poor co-stimulation (Fuchs and Matzinger, Science 258:1156-1159 (1992); Ronchese and Hausmann, J. Exp. Med. 177:679-690 (1993)). Moreover, B lymphocytes lack specific receptor(s) for GM-CSF, hence making it difficult to attribute the effect of the DNA/GM-CSF on the IgM to IgG1 switch to activation of B cells by GM-CSF. On the other hand, GM-CSF is known to enhance both viability and function of DC (Heufler et al., J. Exp. Med. 167:700-705 (1988); Witmer et al., J. Exp. Med. 166:1484-1498 (1987)), activate cells of the dendritic lineage *in vitro* (Inaba et al., *supra*, 1992; Caux et al., Nature 360:258-261 (1992); Scheicher et al., J. Immunol. Methods 154:253-264 (1992); Sallusto and Lanzavecchia, J. Exp. Med. 179:1109-1118 (1994)), and potentiate antigen presentation *in vivo* (Jones et al., Eur. J. Clin. Microbiol. Infect. Dis. S47-53 (1994); Disis et al., Blood 88:202-210 (1996)). The role of DC in the IgM to IgG1 switch was tested using mice carrying a mutation in the *relB* subunit of the NF- γ B complex which lack bone-marrow derived mature DC (Burkly et al., Nature 373:531-536 (1995)).

Mice carrying the *relB* mutation (-/-) were generated as previously described (Burkly et al., *supra*, 1995) and have a number of defects associated with mature dendritic cell function. First, spleen cells from *relB* mutant mice show a very poor ability to stimulate T cells in both allogeneic responses and peptide specific responses (Burkly et al., *supra*, 1995). Second, negative selection of autoreactive T cells in the thymus is impaired (Laufer et al., *Nature* 383:81-85 (1996); DeKoning et al., *J. Immunol.* 158:2588-2566 (1997)). Third, secondary lymphoid tissues fail to develop in mutant mice (Burkly et al., *supra*, 1995; Lo et al., *Am. J. Pathol.* 141:1237-1246 (1992), a feature in part attributed to the absence of mature interdigitating dendritic cells (IDC) that help organize lymphoid tissues. However, antigen presenting function is not entirely absent as T cell and antibody responses can be generated *in vivo* against influenza virus infection (Burkly et al., *supra*, 1995). This residual antigen presenting function is possibly due to macrophages, B lymphocytes, or dendritic cell precursors. Thus, *relB* mutant mice constitute an ideal model system to test the role of DC in isotype switch during STI.

STI was induced in *relB* homozygous mutants (-/-) or hemizygotes (-/+) and in bone-marrow chimeras constructed using mutant bone marrow donors and irradiated normal C57Bl/6 recipients. As seen previously, bone marrow chimeric mice have a less severe form of the mutant syndrome while retaining the impaired antigen-presenting function and immune responsiveness of the homozygous *relB* (-/-) mutant mice (Burkly et al., *supra*, 1995). Moreover, in these mice, germinal center formation appears to be normal and FDC are detected with

normal distribution. By comparing the outcome of STI induced with DNA/GM-CSF to that induced with γ 1NANP DNA, it is possible to determine if DC play any role in the IgM to IgG1 switch. Neither (-/-) bone marrow chimeras nor *relB* mutant produced IgG1 antibodies following inoculation with DNA/GM-CSF (Figure 22A). In contrast, all (-/+) bone marrow chimeras and hemizygous (-/+) mice produced IgG1 antibodies as seen in normal C57Bl/6 mice. Not surprisingly, none of the mice inoculated with γ 1NANP DNA produced IgG1 antibodies (Figure 22B). All bone marrow chimeras, *relB* mutants (-/-) and hemizygotes (-/+) produced IgM antibodies after inoculation with either DNA/GM-CSF or γ 1NANP DNA (Figure 22C and 22D), confirming that they were immunized and ruling out a possible bias introduced by the bone marrow transfer. The immune response in the *relB* mutants and hemizygous mice was generally lower than that detected in bone marrow chimeras but its significance is not clear. Thus, in the absence of DC the effect of GM-CSF on the IgM to IgG1 switch was abrogated.

These results demonstrate that administration of a nucleic acid molecule encoding an epitope fused to GM-CSF promotes the IgM to IgG switch.

EXAMPLE VII

Immunological Memory After Somatic Transgene Immunization is Positively Affected by Priming with GM-CSF and Does Not Require Bone Marrow-Derived Dendritic Cells

This example describes enhanced immunological memory when an administered nucleic acid molecule is primed with GM-CSF.

The protocols used are described below (Gerloni et al., Eur. J. Immunol. 28:1832-1838 (1998)).

Eight to ten week old C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor ME) and were kept in the animal facility of the University of California, San Diego. Bone marrow chimeras were constructed by injecting 7×10^6 bone marrow cells from *relB* (-/-) (Lo et al., *supra*, 1992) or hemizygous (-/+) mice as donors into irradiated (1100R) C57BL/6J recipients as described (Burkly et al., *supra*, 1995). Mice were bred and kept in the animal facility of the Scripps Research Institute. Inoculation with plasmid DNA was performed eight weeks after bone marrow transfer.

The construction and purification of plasmids γ 1NANP (DNA) and γ 1NANP/GM-CSF (DNA/GM-CSF) were generated as described previously in Example VI. Purified plasmids were stored at -20°C until use. Engineered antibody γ 1NANP was produced and purified as described previously (Sollazzo et al., *supra*, 1989; Billetta and Zanetti, *supra*, 1992). A synthetic peptide containing multiple (>10) repeats of the NANP sequence, (NANP) $_n$, was the kind gift of Dr. A. Verdini (Monterotondo, Italy). Peptide DENGNYPLQC of the human invariant chain served as control.

DNA vaccination consisted of a single intrasplenic inoculation of 100 μg of plasmid DNA in 30 μl of sterile saline solution as described in Example I. Mice immunized with the affinity-purified γ 1NANP protein received a subcutaneous injection of the protein (50 $\mu\text{g}/\text{mouse}$) in complete Freund's adjuvant (CFA). Booster injections consisted of either a single subcutaneous injection of affinity-purified γ 1NANP protein (50 μg per

mouse) emulsified in incomplete Freund's adjuvant (IFA),
 or 10^5 irradiated *P. falciparum* sporozoites injected
 intraperitoneally in a 0.4 ml of Dulbecco minimal
 essential medium. Sporozoites were produced in *Anopheles*
 5 *freeborni* mosquitos infected as described (Wirtz et al.,
supra, 1987).

Antibodies to synthetic peptide (NANP)_n and
 γ1NANP were done as in Example II. The isotype of
 10 antibodies was determined using goat antibodies specific
 for the murine IgM and IgG1 classes (Caltag; San
 Francisco CA) (see Example III).

GM-CSF heightens the anamnestic response
 15 induced by antigenized antibody in IFA. The anti-NANP
 response was measured in mice primed with DNA/GM-CSF or
 DNA and subsequently boosted with antigenized antibody
 γ1NANP in IFA. Inoculation of DNA/GM-CSF but not DNA
 induced IgG1 antibodies during the primary response (see
 20 Example VI). A booster injection with antibody γ1NANP in
 IFA increased the IgG1 titer in DNA/GM-CSF primed mice
 and promoted the *de novo* synthesis of IgG1 antibodies in
 mice primed with DNA alone (Figure 23, left panel). In
 two independent experiments, the antibody titer was on
 25 average 4 fold higher (4.1-4.4 vs 3.5-3.8) in mice
 primed with DNA/GM-CSF than in mice primed with DNA alone
 (Table 8). Interestingly, the antibody titer measured in
 mice primed with DNA/GM-CSF was also higher than that of
 control mice primed and boosted with antigenized antibody
 30 γ1NANP in Freund's adjuvant (Figure 23, left panel). The
 titer of IgM antibodies was similar in all groups whether
 GM-CSF had been used or not (Figure 23, right panel).

Table 8. Ig G1 responses in mice primed with DNA/GM-CSF and boosted with antigenized antibody protein.

Experiment No. a) Well coating Primary response Immunogen Secondary response Immunogen

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Experiment No. a)	Well coating	DNA	DNA/GM-CSF	Enhancement (fold)	DNA	DNA/GM-CSF	Enhancement (fold)
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1	NANPn	<200 (2.3) ^{b)}	1.600 (3.2)	8	3.200 (3.5)	12.800 (4.1)	4
2		<200 (2.3)	1.600 (3.2)	8	6.400 (3.8)	25.600 (4.4)	4
1	YNANP	<200 (2.3)	12.800 (4.1)	64	102.400 (5.0)	409.600 (5.6)	4
2		<200 (2.3)	6.400 (3.8)	32	51.200 (4.7)	204.800 (5.3)	4

a) The two experiments represented were run independently. Each group consisted of four mice. Priming was performed by a single intrasplenic inoculation of DNA or DNA/GM-CSF. The booster immunization was given at day 35 with YNANP antibody in IFA. Pooled sera were tested against the synthetic peptide (NANP)n or the whole antigenized antibody as indicated. b) Values refer

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to antibody titers expressed as reciprocal of the last positive dilution. In parentheses are indicated the corresponding log 10 titers.

5 GM-CSF heightens the anamnestic response induced by injection of *P. falciparum* sporozoites. Mice primed by inoculation of plasmid DNA respond to a booster immunization by *P. falciparum* sporozoites with a typical secondary response (see Example III). This was
10 determined to be immunologically relevant since booster by parasites is equivalent to a challenge response or restimulation by infection. Booster by parasites yielded 4 fold higher IgG1 anti-NANP antibody titers in mice primed with DNA/GM-CSF as compared with mice primed with
15 DNA only (Log 4.7 vs. 4.1) (Figure 24, left panel). No antibodies were detected in mice primed with saline and boosted with sporozoites (negative controls). The effect on IgM antibodies was minimal (Figure 24, right panel). This experiment confirms, therefore, that GM-CSF given
20 during priming heightens the IgG1 memory response irrespective of the composition of the antigen used in the booster immunization.

25 GM-CSF heightens the anamnestic response in mice lacking bone marrow-derived dendritic cells. The role of bone marrow derived DC (interdigitating dendritic cells, IDC) in the enhancement of memory IgG1 antibodies was studied in mice carrying the *relB* (-/-) mutation. These mice lack mature IDC and carry a number of defects
30 associated with mature dendritic cell function, including failure to develop secondary lymphoid tissues (Lo et al., *supra*, 1992; Burkly et al., *supra*, 1995). As disclosed in Example VI, *relB* (-/-) mice or *relB* (-/-) chimeras inoculated with DNA/GM-CSF do not produce IgG1
35 antibodies. The present experiments were performed with

$relB$ $(-/-)$ chimeras only since these mice are more resistant and have a less severe form of the mutant syndrome while retaining the impaired antigen-presenting function of the homozygous $relB$ $(-/-)$ mutant mice (Burkly et al., *supra*, 1995). In $relB$ $(-/-)$ chimeras, antibody 33D1-reactive IDC are within relatively normal number (Crowley and Lo, in Targeted gene knockouts: insights into dendritic cell biology, Academic Press, London (1998)) but are functionally inactive (DeKoning et al., *supra*, 1997). Germinal center FDC, which are of host origin, are detected with normal distribution (see Example VI). $RelB$ $(-/+)$ chimeras served as controls.

After booster with antigenized antibody $\gamma 1NANP$ in IFA, the anti-NANP response rose sharply, both in the IgG1 and IgM component, with titers proportionally higher in mice primed with DNA/GM-CSF (Figure 25). The antibody response in $relB$ $(-/+)$ chimeras was comparable to that generated in adult immunocompetent C57BL/6 mice. During priming, $relB$ $(-/-)$ chimeras did not develop anti-NANP antibodies, irrespective of the type of DNA used for immunization or the immunoglobulin isotype considered (Figure 25, Panels A and C). However, lack of primary response in $relB$ $(-/-)$ chimeras was not due to absence of immune responsiveness since these mice produced IgM antibodies against the whole TgIg (Figure 26, panel C). It appears, therefore, that the enhancing effect of GM-CSF on the anamnestic response can not be attributed to a summation effect since, as indicated by the $relB$ $(-/-)$ chimeras, enhancement occurs after booster even though no antibodies are produced during the primary response (Figure 25, panel A; Figure 26, panel A).

These results demonstrate that inclusion of the cytokine GM-CSF enhances the immune response to a B cell epitope administered as a nucleic acid encoding the

epitope. The results also indicate that GM-CSF functions as a modulator of the immune response and immunological memory.

EXAMPLE VIII

Activation of CD4 T Cells by Somatic Transgenesis Induces Generalized Immunity of Uncommitted T Cells and Immunologic Memory

This example describes the activation of CD4 T cells with administration of a nucleic acid molecule encoding an epitope.

The protocols used are described below (Gerlioni et al., J. Immunol. 162:3782-3789 (1999)).

Eight to ten week old C57BL/6 mice were purchased from the Jackson Laboratories (Bar Harbor ME) and were kept in the animal facility of the University of California, San Diego.

Plasmids $\gamma 1NV^2NA^3$ and $\gamma 1NANP$ were engineered as described in Example IV and Sollazzo et al., *supra*, 1990a, respectively. pSV2Neo is the original plasmid forming the backbone of the pNeogl vector without the human $\gamma 1$ C region gene. Plasmid DNAs were purified using a Qiagen MEGAPREP kit (Qiagen, Chatsworth, CA). This plasmid was used as a control in the immunization experiments. The purity of the DNA was monitored using the following equation: $\%N = (11.1R - 6.32) / (2.16 - R)$ where $R = 260nm / 280nm$, $\%N = \% \text{ of Nucleic Acid}$. Purified plasmids were stored at $-20^\circ C$ until use.

Recombinant antigenized antibodies $\gamma 1NV^2NA^3$ and $\gamma 1NANP$ were produced in transfectoma cells and purified as described in Example IV (Sollazzo et al., *supra*, 1990a). Synthetic peptides NANPNANPNANP and

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NANPNVDPNANP were synthesized in the Peptide Chemistry Core Facility of the University of California, San Diego.

Mice were inoculated intraspleen with 100 γ g of plasmid DNA in 50 μ l of sterile saline solution as previously described in Example I. Booster injections were administered on day 90, 110, 120 and 150 after priming by a single subcutaneous injection (50 μ g per mouse) of affinity-purified γ 1NV²NA³ antibody emulsified in incomplete Freund's adjuvant (IFA).

After animals were inoculated, at the time of harvest, mice were sacrificed and the lymph nodes and spleens removed, and crushed in a tissue shredder to remove excess tissues and release cells. Single cell suspensions were treated with red blood cell lysis buffer (Sigma; St. Louis, MO) and cultured (10^6 cells/ml) in RPMI 1640 medium (Irvine Scientific; Santa Ana CA) supplemented with Hepes buffer, glutamine, 7.5% fetal calf serum and 50 μ M 2-mercaptoethanol, in the presence or absence of synthetic peptides NANPNVDPNANP or NANPNANPNANP (50 μ g/ml) in triplicate. The cells were incubated at 37°C in 10% CO₂ for 3 days. (³H)-Thymidine was added at 1 μ Ci/well and the cells were incubated for 16-18 hours at 37°C. Cells were harvested onto glass fiber filter mats using a Tomtec cell harvester and the radioactivity was measured in a liquid scintillation counter (Betaplate; Wallac; Tuku Finland). Results are expressed as Stimulation Index (S.I.) calculated as the ratio of (counts per minute of cells cultured in the presence of synthetic peptide)/(counts per minute of cells cultured in the absence of peptide). Concanavalin A (ConA) stimulation was used as a polyclonal activator and positive control.

CD4⁺ and CD8⁺ T cells were isolated by antibody plus complement-mediated depletion from splenocytes of

mice immunized 7 days earlier by DNA inoculation. Briefly, cell suspensions (30×10^6 cells/ml) were treated with monoclonal antibody to CD8 (3.155) or CD4 (RL172) for 30 minutes on ice. After washing, anti-T cell antibodies were cross-linked with a mouse anti-rat (MAR 18.5) monoclonal antibody for 30 minutes on ice and rabbit complement was added twice for 30 minutes at 37°C . The cell suspension was then washed twice and resuspended at the concentration of 5×10^6 cells/ml in RPMI (Irvine Scientific). The purity of the separated cell fractions was assessed by analysis on a FACScan with Cellquest software (Becton&Dickinson, Mountain View, CA) at the Flow cytometry facility of The La Jolla Institute for Allergy and Immunology, using phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 monoclonal antibodies (Pharmingen, San Diego, CA).

Culture supernatants were harvested 40 hours after initial seeding and were stored at -20°C . The supernatants from three separate triplicate cultures were pooled for each mouse. IL-2 activity was determined in a bioassay utilizing the IL-2- and IL-4-dependent NK.3 cells in the presence of anti-IL4 (purified from the 11B11 cell line, ATCC). Briefly, 100 μl (1:2 dilution in medium) of 40 hour culture supernatants were added in duplicate to 100 μl of NK.3 cells ($10^6/\text{ml}$) and incubated for 36 hours. (^3H)-Thymidine was added at 1 $\mu\text{Ci}/\text{well}$ during the last 12 hours. Cells were harvested as specified above. Results are expressed as counts per minute.

IL-4, IL-5 and IFN- γ were measured in the same 40 hours culture supernatants by ELISA as described previously using the antibodies 11B11 and biotinylated anti-IL-4 (BVD6, Pharmingen), TRFK5 and biotinylated TRFK4 and R46A-2 and biotin-XMG1.2 (Pharmingen), respectively.

Standard curves were constructed with purified IL-2, IL-4, IL-5 and IFN-g (supernatants from the respective X63.Ag. cell lines). Tests were done in duplicate.

5 The presence of transgenic Ig in the serum of mice was detected using a capture enzyme-linked immunosorbent assay (ELISA) as described in Example I.

10 Antibodies to transgenic Ig (γ 1NANP) were detected by ELISA as in Example III. The isotype of antibodies was determined by ELISA in Example III.

15 As a source of antigen presenting cells (APC), spleen cells from unprimed mice were used and cultured with LPS/Dextran (25 μ g/ml) for 24 hours and treated for 30 min at 37°C with 25 μ g/ml mitomycin C (Sigma). Before use, spleen cells from naive, primed, or primed and boosted mice were mixed with 2×10^6 /ml APC in 96-well flat-bottom plates in the presence of 50 μ g/ml synthetic peptide (-NVDP-). Each dilution of cells was plated in replicates of 48. Supernatants were harvested after 36 hours and 20 μ l from each culture was tested for IL-2 activity using the NK.3 cell line. Single cultures supernatants were considered positive when the value of 3 H-thymidine incorporation was greater than the mean of the replicate control cultures with no antigen plus two standard deviations. Frequencies of cytokine producing cells were calculated using the program described by Waldman and were calculated using maximum likelihood analysis.

35 The effects of STI on activation of T lymphocytes was determined. T cell responses were assessed using DNA coding for 12 amino acid determinants of the circumsporozoite (CS) protein of *Plasmodium falciparum* malaria parasite. The plasmid γ 1NV²NA³ DNA

contains an Ig H chain gene in which the V domain is engineered to code for a Th cell determinant (NANPNVDPNANP) in CDR2 and a B cell epitope (NANPNANPNANP) in CDR3 (antigenized antibody) . The Th cell determinant (-NVDP-) and the B cell epitope only differ by two amino acid residues, A to V and N to D in position 5 and 6, respectively. As disclosed in Example IV, an antigenized antibody product of the same gene, when injected in complete Freund's adjuvant, induces specific T cell proliferation and IL-2 secretion.

Spleen cells harvested 7 days after a single intraspleen inoculation of 100 μ g of γ 1NV²NA³ DNA proliferated in culture after re-stimulation with the antigenized antibody expressing the Th cell determinant or the corresponding 12mer Th cell determinant peptide (Figure 27A). Proliferation occurred when cells were cultured with the T- but not the B-cell peptide demonstrating specific activation by the heterologous peptide in CDR2. Proliferation after culture with the antigenized antibody expressing -NVDP- also suggests that the CDR2 peptide within the antibody molecule is processed and presented by APC. When compared with the proliferative response of cells from mice immunized with the antigenized antibody in CFA, STI induced a response of similar or greater magnitude. Specific activation of T cells was accompanied by marked production of IL-2 (Figure 27B). The lower amounts of IL-2 measured in cultures re-stimulated *in vitro* with the -NVDP- peptide most likely reflect a higher consumption as cells in these cultures were proliferating to a greater extent.

The kinetics of T cell activation *in vivo* was monitored in another experiment in which spleen cells were harvested 3, 7, 14 and 21 days after DNA inoculation (Figure 28). Proliferation was absent on day 3, peaked on day 7, and was minimal by day 21. The

response was specific, measured only when cells were re-stimulated *in vitro* with the Th cell determinant peptide. The production of IL-2 paralleled the proliferative response.

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Splenocytes harvested on day 7 and 14 were also assayed for production of IFN- γ , IL-4 and IL-5 to assess whether any polarization to Type 1 and Type 2 phenotype had occurred (Figure 29). Both IFN- γ and IL-4 were detected, albeit in different amounts and IL-5 was absent. Since IFN- γ specific activity is on average 100 fold lower than IL-4, and IL-4 is typically secreted in much lower quantities than IFN- γ , these results indicate that both cytokines are produced proportionally and that cells activated through STI remain, by and large, uncommitted.

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Activated cells were determined to be CD4⁺ T lymphocytes. CD4⁺ T cells were formally identified as the cell population proliferating and making cytokines. Spleen cells from mice immunized 7 days earlier were depleted of CD4⁺ and CD8⁺ cells by treatment *in vitro* with monoclonal antibodies specific for CD8 or CD4 plus complement. By flow-cytometry the purity of the two populations was 94 % (CD4) and 99 % (CD8), respectively (Figure 30C and 30D). The two cell populations were then cultured *in vitro* with the addition of fresh APC from naïve mice and synthetic peptide -NVDP-. Proliferation occurred in the CD4⁺ but not in the CD8⁺ T cell population (Figure 30E). Similarly, IL-2 production was detected only in the CD4⁺ T cell population (Figure 30F). These results demonstrate that STI selectively activates CD4⁺ T lymphocytes.

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T cell immunity was found to spread to other secondary lymphoid organs. Germane to the present studies was to determine the extent to which priming

induces generalized T cell activation. In a first set of experiments, spreading of immunity to other secondary lymphoid organs was monitored by measuring cell proliferation and IL-2 production in a pool of inguinal, mesenteric and cervical lymph node cells. Seven days after DNA inoculation cells of the lymph node pool proliferated specifically upon re-stimulation *in vitro* with the -NVDP- but not with the B-cell epitope peptide (Figure 31A). When compared with spleen cells, proliferation in lymph nodes was of a lesser magnitude. On day 14, the magnitude of the response in lymph node cells increased markedly reaching values comparable to spleen cells. On day 21, only residual proliferative activity existed in both lymph node and spleen cells. The magnitude and specificity of the proliferative responses were reflected by the levels of IL-2 in the corresponding culture supernatants (Figure 31B). These kinetic analyses revealed that T cell activation in lymph nodes parallels that in the organ in which the process of immunity was initiated.

Analysis of the tempo of these responses in relation to other parameters of STI revealed something interesting. When the ratio between the stimulation indexes in lymph nodes and spleen was calculated, it became evident that, by day 14, T cell responsiveness in lymph nodes was prevalent. Moreover, the peak of the proliferative response in lymph nodes appeared to correlate with the peak values of transgenic Ig in the serum (Figure 31C). The results indicate that a pattern of proportionality exists between secretion of transgenic Ig and spreading of T cell immunity.

Whether pooled lymph node cells were a true representation of a generalized response was further analyzed in lymph nodes collected according to precise anatomical distribution, lower (popliteal, caudal,

sciatic and lumbar), middle (mesenteric, renal and epigastric) and upper (axillary, brachial, deep and superficial cervical) lymph nodes. T cell proliferation and IL-2 production were measured 14 days after DNA inoculation (Figure 31D and 31E). As shown both parameters were comparably elevated in all three lymphoid districts.

The effects of linked recognition of Th and B cell epitopes on the antibody response was determined. Expression of B and Th cell epitopes in linked association in transgenic Ig is expected to produce quantitative and qualitative effects on the B-cell response. First, antibody titers during priming were determined. Mice given the transgene coding for both the Th cell determinant and the B-cell epitope produced consistently higher antibody titers than mice immunized with the B-cell epitope-containing gene (Figure 15), a result in agreement with the results described in Example IV. Second, specific activation of Th cells by the -NVDP- determinant was determined to be sufficient to promote the IgM to IgG1 switch. As disclosed in Example I and VI, STI mice produce mainly IgM, low level IgG2a, but no IgG1 unless the transgene is appropriately manipulated to increase the activation of dendritic cells (see Example VI). Mice given the Th/B double-epitope transgene developed IgM and IgG1 antibodies (Figure 32). Presence of the Th cell determinant in the transgene did not affect the IgG2a response, which was minimal in both groups. These results indicate that the concomitant local activation of CD4⁺ T cells and B lymphocytes drove secretion of downstream cytokines required for isotype switch in B cells. T cell immunity triggered by the Th cell determinant in linked association with a B-cell epitope optimizes the B-cell response by heightening the antibody titer and by promoting isotype switch. Since similar levels of IgM and IgG2a were observed

irrespective of the presence of the Th cell determinant, the increased amount of specific antibodies is likely due to IgG1 antibodies.

5 The response to secondary exposure to antigen *in vivo* was determined. Frequencies were determined in mice given a booster immunization with antigenized antibody γ 1NV²NA³ (50 μ g) in incomplete Freund's adjuvant (IFA) 90-110 days after DNA priming. In light of the
10 fact that memory T cells are highest in number four days after booster immunization, LDA was performed in spleen cells harvested at this time.

 The frequency of antigen-responsive T cells was
15 much higher after booster immunization. The effect was not merely due to expansion of specific T cells by immunization with protein antigen in IFA because, in the absence of DNA priming, the frequency was about three times lower. For comparative purposes, LDA studies were
20 also performed 4 and 7 days after single DNA inoculation (Table 9). On day 4 and 7 the frequency was 1/90,200 (group II) and 1/50,500 (group III), respectively. Four days after priming with protein antigen in IFA, the frequency was 1/60,000 (group VII). The average
25 frequency during the memory response was 1/21,900 that is 2.5-4 times higher. Table 9 also shows that early after DNA priming antigen-responsive T cells were enriched 75 fold over naive precursors but dropped to 1/424,500 (group V) by day 110. Collectively, these results
30 indicate that priming by STI establishes T cell memory. Re-encounter with antigen induced a faster and higher specific response.

Table 9. Frequency of CD4 T cells specific for the Th determinant.

Group	Priming	Days After Priming	Booster	Day of Booster	Frequency of CD4 cells ^a
I	None				1/1,558,000 ^a
II	DNA	4			1/90,200
III	DNA	7			1/50,500 ^a
IV	DNA	14			1/36,400
V	DNA	110			1/424,500 ^b
VI	DNA		Protein	110	1/21,900 ^{b,c}
VII	None		Protein		1/60,000 ^b

^a Values represent the average of two independent experiments.

^b Values represent the average of three independent experiments. The booster immunization was performed on day 90-110.

^c Spleen cells were harvested and put in culture 4 days after booster immunization.

The level of cytokines produced by re-activated memory T are illustrated in Table 10. IFN- γ was detected in half (2/4) of the animals, IL-4 was produced in all four instances and IL-5 was detected in two cases only.

Table 10. Cytokines in the supernatant of memory cell cultures.

Mouse	Priming	Day of Booster	Type of Booster	IFN- γ (ng/ml)	IL-4 (pg/ml)	IL-5 (ng/ml)
1	DNA	150	Protein	7.7	230	ND
2	DNA	150	Protein	ND	240	3.2
3	DNA	150	Protein	59.9	440	7.3
4	DNA	150	Protein	ND	190	ND

ND, not detected.

Spreading of T cell responsiveness from the spleen to lymph nodes throughout the body is an original feature of the results disclosed herein. As a rule, immunization with antigen in adjuvant activates specific T cells only in the lymph nodes proximal to the site of injection. Recent studies using adoptive transfer of TCR-transgenic T cells clearly showed that subcutaneous immunization with antigen in adjuvant attracts specific T cells only in the draining but not in the non-draining lymph nodes. Thus, under conventional immunization procedures, migrating T cells are sequestered in the draining lymph nodes by antigen transported by dendritic cells or macrophages via the lymphatics. As disclosed herein, T cell responsiveness in distal lymph nodes began approximately at the same time (day 7) as in the spleen and peaked on day 14. Maximal T cell responsiveness occurred when transgenic Ig were most abundant in the serum. The results disclosed herein support a model in which transgenic Ig are released into the circulation, undergo localization in the cortex of distal lymph nodes, and serve as an anchor for T cells. Whether T cells activated in lymph nodes derive from re-circulating effector T cells or from naive CD4⁺ T lymphocytes undergoing *de novo* activation, is not known. The first possibility is plausible since the observed kinetics are consistent with the notion that effector T cells leave the site where they encountered antigen within 48 hours

and re-circulate through the body in 24 hours . The second possibility, *de novo* activation, is consistent with the fact that antigenized Ig clearly supported T cell activation *in vitro*.

5

The results disclosed herein indicate that STI is an effective way to activate CD4 T cells and establish durable T cell memory. The frequency of antigen-reactive T cells increased 3-4 fold in a long term primed animal and again several fold after booster immunization. In addition, the response was faster than the primary response, consistent with a functional definition of immunologic memory . In all likelihood, early effector T cells gave rise to resting memory cells, which are known to re-circulate as a pool through spleen and lymph nodes until they are sequestered again by antigen 24-48 hours later. The cytokines produced by re-activated memory T cells did not follow the pattern observed during priming. IFN- γ was detected in half (2/4) of the animals, IL-4 was produced in all four instances and IL-5 was detected in two cases only. These results indicate that the characteristics of priming are not maintained during the memory response and that priming does not pre-commit the outcome of a subsequent booster immunization.

25

These results demonstrate that administration of a nucleic acid molecule encoding an epitope results in T cell responsiveness with long term immunologic memory.

30

35

EXAMPLE IX

Activation of CD8 T Cells by Somatic Transgenesis Induces
Protection Against Challenge with Influenza Virus

5

This example describes the activation of CD8 T cells with administration of a nucleic acid molecule encoding an epitope from the influenza virus A/PR8.

10

The protocols used are in part described below (Billetta et al., Eur. J. Immunol. 25:776-783 (1995)).

15

Eight to ten week old C57BL/6 (H-2b) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained in the animal facility of the University of California, San Diego, throughout the duration of the experiments.

20

A H-chain gene was engineered to express in the third complementarity-determining region (CDR3) 13 amino acid residues from the sequence of the A/PR/8/34 influenza virus nucleoprotein (NP) antigen (Figure 33). This NP peptide is presented in association with the Db allele in H-2b mice.

25

The NP synthetic peptide ASNeNMeTM (amino acid residues 366-374) was purchased from Multiple Peptide System (San Diego, CA) and synthesized on an ABI 430-A automated synthesizer (Applied Biosystems, Inc., Foster City, CA).

30

35

Mice were inoculated with 100 µg of plasmid DNA per inoculation. All DNA inoculations were done intraspleen as indicated under Example I. Groups of mice were additionally boosted after 12 sddks with 50 µg of NP peptide emulsified in incomplete Freund's adjuvant. Control groups consisted in mice immunized twice with 50

µg of NP peptide emulsified in concomplete Freund's adjuvant (positive control) or mice of the same age group that did not receive any treatment (negative control).

5 Mice were challenged intranasally with 10xLD50 dose of infectious homologous virus. After challenge mice were monitored for loss of weight and survival.

10 Cytotoxicity was tested on spleen cells using a 4 hour ⁵¹Cr release assay. Briefly, RMA-S (H2b) target cells were labeled with Na⁵¹CrO₄ (150 mCi/1 x 10⁶ cells) for 1 hour at 37°C in an atmosphere of 5% CO₂ with or without NP peptide (10µg/ml), then washed and resuspended in culture medium supplemented with 10% FCS. One hundred
15 µl of ⁵¹Cr-labeled target cells (2.5 x 10⁵ cells/ml) were mixed with effector cells in 100 µl at various (100:1) effector:target (E:T) ratio. The plates were incubated for 4 hours at 37°C in 5% CO₂, then centrifuged at 500 g for 4 minutes. One hundred µl of supernatant were
20 removed and counted in a gamma counter. Spontaneous and maximal ⁵¹Cr releases were determined by incubating target cells in medium alone or in the presence of 1% Triton 100x, respectively. Percent cytotoxicity was calculated from triplicate wells as follows:
25
$$\frac{[\text{experimental release} - \text{spontaneous release}]}{[\text{maximal release} - \text{spontaneous release}]} \times 100.$$

30 Early studies *in vitro* demonstrated that a B cell harboring an If H chain transgene process and present in a T cell peptide to cytotoxic (CD8) T cells, and are lysed with high efficiency (Billetta et al., Eur. J. Immunol. 25:776-783 (1995)). For instance, when B-lymphoma cells (Db) were transfected with the H chain gene engineered to express in the third DCR the NP
35 peptide ASNENNETMESSTL they were efficiently killed by specific CTL in a dose-dependent manner indicating that processing the presentation of the NP peptide at the

surface of the cell had occurred. Killing was MHC-restricted and blocked by an anti-class-I MHC monoclonal antibody.

5 In a series of experiments, it was shown that C57BL6 mice inoculated with this transgene develop a CTL response. Spleen cells from inoculated mice were harvested three weeks after immunization and tested for their ability to kill NP peptide-pulsed RMA-S target cells
10 in a conventional cytotoxicity assay. RMA-S cells without peptide served as a control. In this assay we found that between 60--75% of mice had generated a cytotoxic T cell response specific for the influenza NP peptide.

15 Protection and induction of memory CTL was also documented (see Figure 34). In the experiment shown, mice (10 per group) were vaccinated wither via STI or with synthetic peptide in incomplete Freund's adjuvant. A group of mice remained untreated and served as control.
20 Three months after vaccination mice received an intranasal challenge with 10xLD₅₀ dose of infectious influenza virus (i.e. 10 times the lethal dose of 50% of mice). As shown, all untreated mice vaccinated with synthetic peptide in adjuvant died by day 11. As shown,
25 the majority (50 and 60%) of mice vaccinated by somatic transgene immunization survived.

EXAMPLE X

30 Positive Reciprocal Regulation Between Two Th Cell Epitope During Somatic Transgene Immunization

This example describes the activation *in vivo* of CD4 T cells against determinants of a tumor antigen
35 per se unable to induce a cellular response. This is obtained by immunization with nucleic acid molecule

encoding tumor epitopes in linked association with a dominant T cell epitope of the malaria parasite.

Eight to ten week old D57BL/6 (H-2b) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were maintained in the animal facility of the University of California, San Diego, throughout the duration of the experiments.

Two H-chain genes were engineered to express in the CDR3 two amino acid sequences (VTSAPDTRPAP and DTRP3) from the tandem repeat of the tumor antigen MUC-1 (Gendler et al., Proc Natl Acad Sci USA, 84:6060-6064 (1987)). Each gene coding for a single epitope of the MUC-1 antigen was also engineered to code in the CDR2 for the Th cell determinant NANPNVDPNANP from the outer coat of the malaria parasite *P. Falciparum* (Nardin et al., Science 246:1603-1606 (1989)). The corresponding plasmid vector is termed γ 1NV2VTSA3 (Figure 35) and γ 1NV2DTRP3.

Pilot experiments had shown that plasmid DNA coding for just the MUC-1-derived peptide sequence were unable to induce a proliferative response in vivo. However, when plasmids γ 1NV2VTSA3 and γ 1NV2DTRP3 were used, a strong response against the respective MUC-1 epitope was measured (see Figure 35). None of the eight mice immunized with DNA coding for the single MUC-1 epitope alone developed a T cell response. In converse a response occurred in all (10+10) mice immunized with a gene coding in linked association for the MUC-1 epitope and the heterologous Th cell determinant from the malaria parasite (Figure 1).

These results indicate that weak immunogenic epitopes (such as the ones from MUC-1) can be rendered immunogenic by association with a strong heterologous Th-cell determinant. This finding is relevant for the

development of a MUC-1-based vaccine but also for the development of T cell immunity against other tumor antigens. T cells against determinants on tumor antigens are thought to be eliminated in great part during negative selection in the thymus. This leaves the adult organism with only T cells reactive against a minority of determinants, usually weak (cryptic or subdominant) determinants, the residual T cell repertoire. Since the objective of any effort to develop effective strategies of immune therapy against cancer is to expand those residual tumor-specific precursor T cells, it appears that a linked Th/Th association in a gene used for somatic transgene immunization can render immunogenic an otherwise poorly or non-immunogenic Th cell determinant. The significance of this finding reaches beyond tumor antigens, and is applicable to vaccines against self antigens and antigens of exogenous pathogens.

EXAMPLE XI

Somatic Transgenesis Functions *in vitro* for Human B Cells

This example describes the spontaneous transfection of human B cells using bacterial plasmid DNA coding for an immunoglobulin gene.

Raji (MHC class II⁺) and RJ2.2.5 (a MHC class II⁻ variant) were cultured in RPMI-1640 containing 10%FCS supplemented with 2% glutamine.

Plasmid DNA g1NANP was described in Figure 1 and Example I. PCR and RT-PCR methodologies are as described in Example II.

Raji (MHC Class II⁺) and RJ2.2.5 (a MHC class II⁻ variant) were harvested and washed thoroughly with sterile saline, counted and redistributed at various concentrations in 300 μ l of phosphate buffered saline. 5 μ g of plasmid DNA was added to the cell suspension and incubated at 37 °C, for 1 hour in a 5% CO₂ atmosphere. After the incubation the cells were washed with saline and put in culture medium and grown at 37 °C, 5% CO₂. Uptake and transfection were assessed on cells harvested 24 hours later. Genomic DNA was extracted using the QIAamp Blood Kit (Qiagen) and subject to two-round of nested PCR using VDJ specific primers (see Example II). The PCR products were analyzed on a 1% agarose gel with ethidium bromide stain. After 24 hours the transgene was detected with PCR in both the Raji and RJ2.2.5 cells (see Figure 37) suggesting that uptake and integration of the transgene had occurred. Next, we determined that transcription had also occurred. The total RNA of 10⁵ transfected cells was extracted in a single-step after 7 days of culture using granidinium thicyanate phenol-chloroform. By RT-PCR RNA coding for the H chain transgene product was detected in transfected Raji but not in untransfected Raji cells (see Figure 37). A corresponding murine transfectoma cell line was used as a positive control.

These data constitute evidence that human B cells the transgene is are capable of taking up bacterial plasmid spontaneously, and translocate the mammalian gene contained in the plasmid to the nucleus for integration and transcription. This in vitro experiment recapitulates, therefore, the essence of somatic transgenesis as documented in vivo in the mouse.

Throughout this application various publications have been referenced. The disclosures of these publications in their entirety are hereby

I claim:

1. A method for stimulating an immune
response, comprising administering to a lymphoid tissue a
5 nucleic acid molecule comprising an expression element
operationally linked to a nucleic acid sequence encoding
one or more heterologous epitopes.

2. The method of claim 1, wherein said
10 lymphoid tissue is selected from the group consisting of
spleen, lymph nodes, mucosa-associated lymphoid tissue
(MALT), tonsils, Payer's patches, nasal-associated
lymphoid tissue (NALT), Waldeyer's ring, and urogenital
lymphoid tissue.

3. The method of claim 1, wherein said
15 expression element comprises a hematopoietic cell
expression element.

4. The method of claim 3, wherein said
20 expression element functions in a cell selected from the
group consisting of B cell, T cell, and dendritic cell.

5. The method of claim 1, wherein said epitope
25 stimulates an antibody response.

6. The method of claim 1, wherein said epitope
stimulates a CD4 T cell response.

7. The method of claim 1, wherein said epitope
30 stimulates a CD8 T cell response.

8. The method of claim 1, wherein said
35 epitope stimulates a CD4 T cell response and a CD8 T cell
response.

5

10

15

20

25

30

3 E

17. The method of claim 12, wherein said immunoglobulin molecule comprises a light chain.

18. A nucleic acid molecule comprising a hematopoietic cell expression element operationally linked to a nucleic acid sequence encoding a heterologous polypeptide.

5

19. The nucleic acid molecule of claim 18, wherein said expression element functions in a cell selected from the group consisting of B cell, T cell, and dendritic cell.

10

20. The nucleic acid molecule of claim 18, wherein said nucleic acid sequence encodes a polypeptide expressed as a fusion with a cytokine.

15

21. The nucleic acid molecule of claim 20, wherein said cytokine is selected from the group consisting of granulocyte-macrophage colony-stimulating factor, interleukin-2, interleukin-4, interferon- γ , interleukin-5, interleukin-6, interleukin-10 and interleukin-12.

20

22. A nucleic acid molecule comprising a hematopoietic cell expression element operationally linked to a nucleic acid sequence encoding one or more heterologous epitopes, wherein said nucleic acid sequence encodes an immunoglobulin molecule containing said one or more epitopes and wherein said one or more epitopes is inserted within a complementarity-determining region (CDR) of said immunoglobulin molecule.

25

30

23. The nucleic acid molecule of claim 22, wherein said immunoglobulin comprises a variable region.

35

24. The nucleic acid molecule of claim 23, wherein said variable region is a heavy chain variable region.

25. the nucleic acid molecule of claim 23,
wherein said variable region is a light chain variable
region.

5 26. The nucleic acid molecule of claim 22,
wherein said one or more epitopes is inserted in two
CDRs.

10 27. The nucleic acid molecule of claim 22,
wherein said epitope is expressed as a fusion with a
cytokine.

15 28. The nucleic acid molecule of claim 27,
wherein said cytokine is selected from the group
consisting of granulocyte-macrophage colony-stimulating
factor, interleukin-2, interleukin-4, interferon- γ ,
interleukin-5, interleukin-6, interleukin-10 and
interleukin-12.

20 29. A method of treating a condition,
comprising administering a nucleic acid molecule
comprising a hematopoietic cell expression element
operationally linked to a nucleic acid sequence encoding
a heterologous polypeptide, wherein said nucleic acid
25 molecule is targeted to a hematopoietic cell.

30 30. The method of claim 29, wherein said
hematopoietic cell is a B cell.

35 31. The method of claim 29, wherein said
hematopoietic cell is targeted *in vivo*.

 32. The method of claim 29, wherein said
hematopoietic cell is targeted *ex vivo*.

 33. A method of treating a condition,
comprising administering a nucleic acid molecule

comprising a hematopoietic cell expression element
operationally linked to a nucleic acid sequence encoding
a heterologous polypeptide selected from the group
consisting of hormone, cytokine, clotting factor and
immunoglobulin.

5

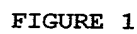
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ABSTRACT OF THE INVENTION

The invention provides a method for stimulating an immune response by administering to a lymphoid tissue a nucleic acid molecule comprising an expression element operationally linked to a nucleic acid sequence encoding one or more heterologous epitopes. The heterologous epitope can be inserted into a complementarity-determining region of an immunoglobulin molecule. The invention also provides a nucleic acid molecule comprising a hematopoietic expression element operationally linked to a nucleic acid sequence encoding a heterologous polypeptide. The invention additionally provides a method of treating a condition by administering a nucleic acid molecule comprising a hematopoietic cell expression element operationally linked to a nucleic acid sequence encoding a heterologous polypeptide, wherein the nucleic acid molecule is targeted to a hematopoietic cell.

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Figure 1. Schematic representation of the experimental design. The subjects were divided into two groups: the control group (CG) and the experimental group (EG). The CG was divided into two subgroups: the control group (CG) and the control group (CG). The EG was divided into two subgroups: the experimental group (EG) and the experimental group (EG). The subjects were divided into two groups: the control group (CG) and the experimental group (EG). The CG was divided into two subgroups: the control group (CG) and the control group (CG). The EG was divided into two subgroups: the experimental group (EG) and the experimental group (EG).



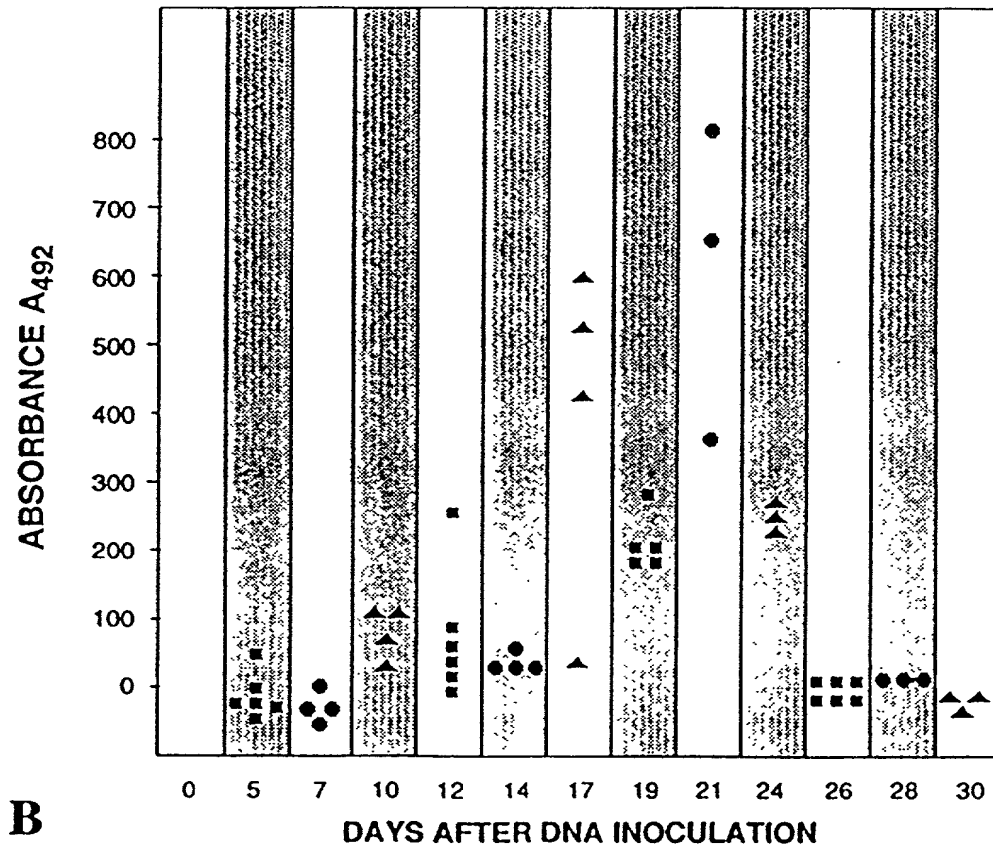
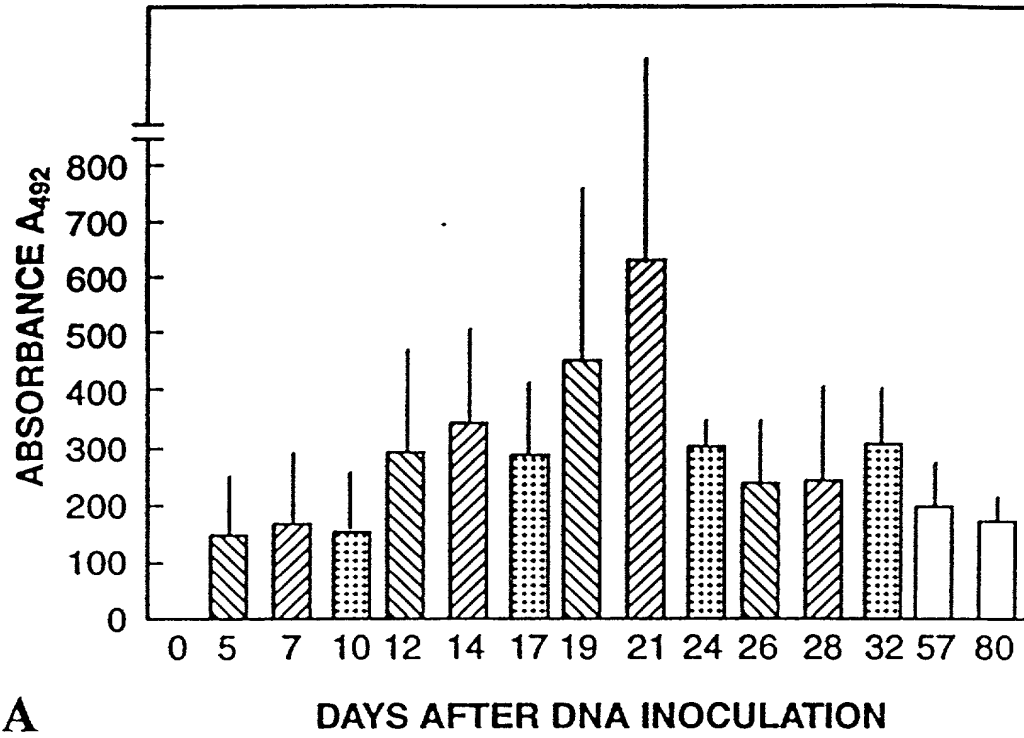


FIGURE 2

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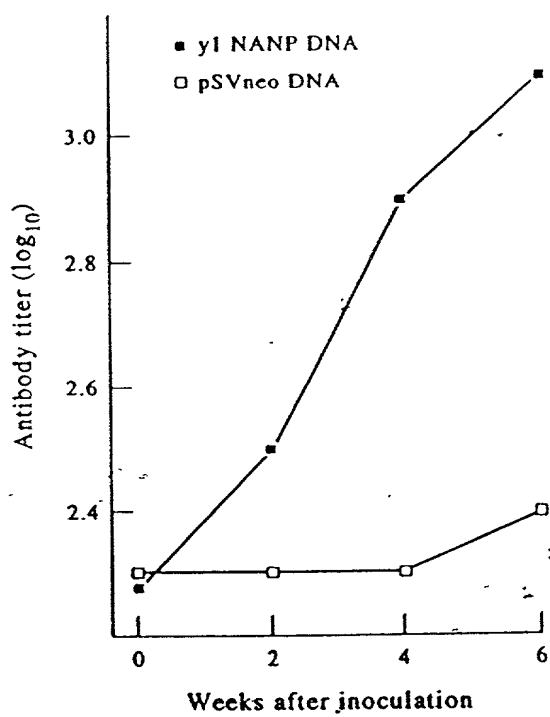


FIGURE 3

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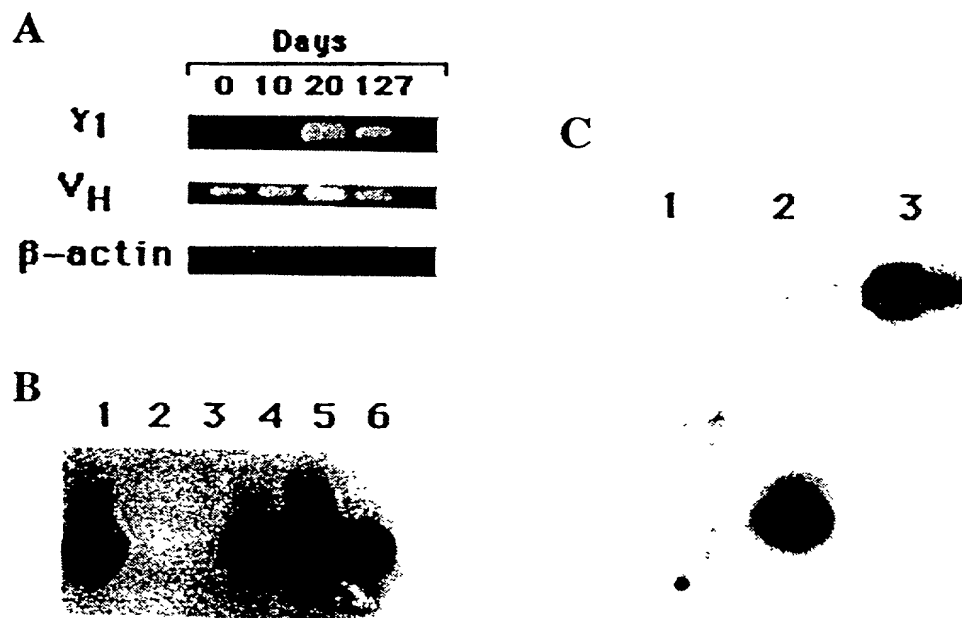


FIGURE 4

FR1

Y1WT-TAC	CACCTGAAGC	TGCTGGAGTC	TGGGGGAGGC	TTAGTGAAGC	TTGGAGGGTC	CCTGAAGCTC	TCTGTGCAG	CCTGTGCATT	CACCTTCAGT
SP7	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP8	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP9	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP10	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP11	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP12	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR35	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR36	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR37	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR38	-----	-----	-----	-----	-----	-----	-----	-----	-----

CDR1

FR2

CDR2

Y1WT-TAC	AGGTATTACA	TGCTTGGGT	TGCGCAGACT	CCAGAGAAGA	GGCTGGAGTT	GGTGGCAGCC	ATTAATAGTA	ATGGTGGTAG	CACCTACTAT
SP7	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP8	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP9	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP10	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP11	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP12	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR35	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR36	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR37	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR38	-----	-----	-----	-----	-----	-----	-----	-----	-----

FR3

Y1WT-TAC	CCAGACACTG	TGAAGGGGCG	ATTCAACATC	TCAGAGACA	ATGCCAAMAA	CACCTGTAC	CTGCAATGA	GCAGTCTGAA	GTCTGAGGAC
SP7	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP8	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP9	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP10	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP11	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP12	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR35	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR36	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR37	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR38	-----	-----	-----	-----	-----	-----	-----	-----	-----

CDR3

FR4

Y1WT-TAC	ACAGCCTTGT	ATTACTGTGC	AAGAAAGCTA	CCCTACTCTC	ATGGTATGCA	CTACTGGGGT	CAAGGAACCT	CAGTCACCGT	CTCCTCAGGT
SP7	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP8	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP9	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP10	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP11	-----	-----	-----	-----	-----	-----	-----	-----	-----
SP12	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR35	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR36	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR37	-----	-----	-----	-----	-----	-----	-----	-----	-----
TR38	-----	-----	-----	-----	-----	-----	-----	-----	-----

Y1WT-TAC	AAGAATGGCC	TCTCCAGGTC	TTTATTTTAA	ACCTTTGTTA	TGGAGTTTTC	TGAGCAATTGC	AG
SP7	-----	-----	-----	-----	-----	-----	-----
SP8	-----	-----	-----	-----	-----	-----	-----
SP9	-----	-----	-----	-----	-----	-----	-----
SP10	-----	-----	-----	-----	-----	-----	-----
SP11	-----	-----	-----	-----	-----	-----	-----
SP12	-----	-----	-----	-----	-----	-----	-----
TR35	-----	-----	-----	-----	-----	-----	-----
TR36	-----	-----	-----	-----	-----	-----	-----
TR37	-----	-----	-----	-----	-----	-----	-----
TR38	-----	-----	-----	-----	-----	-----	-----

FIGURE 5

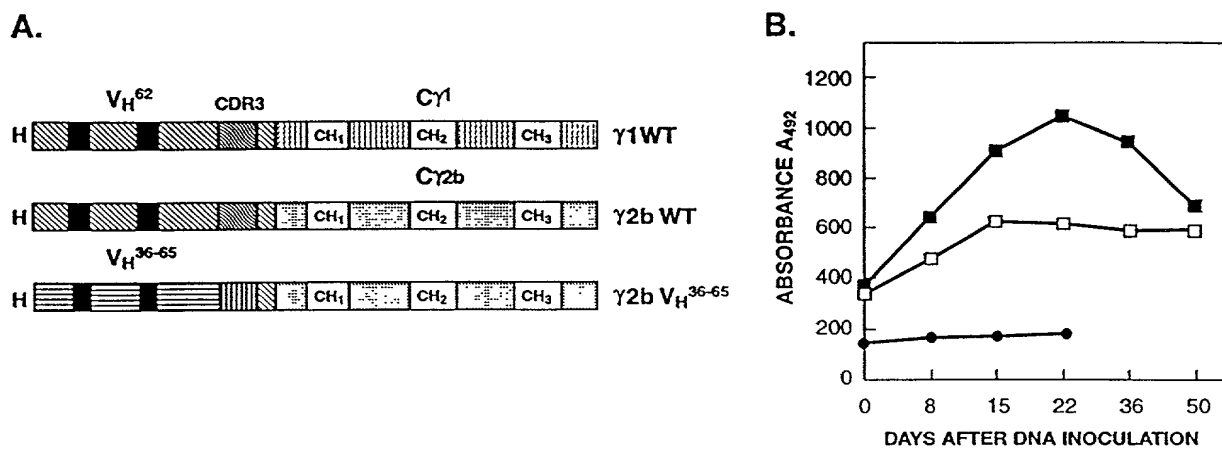


FIGURE 6

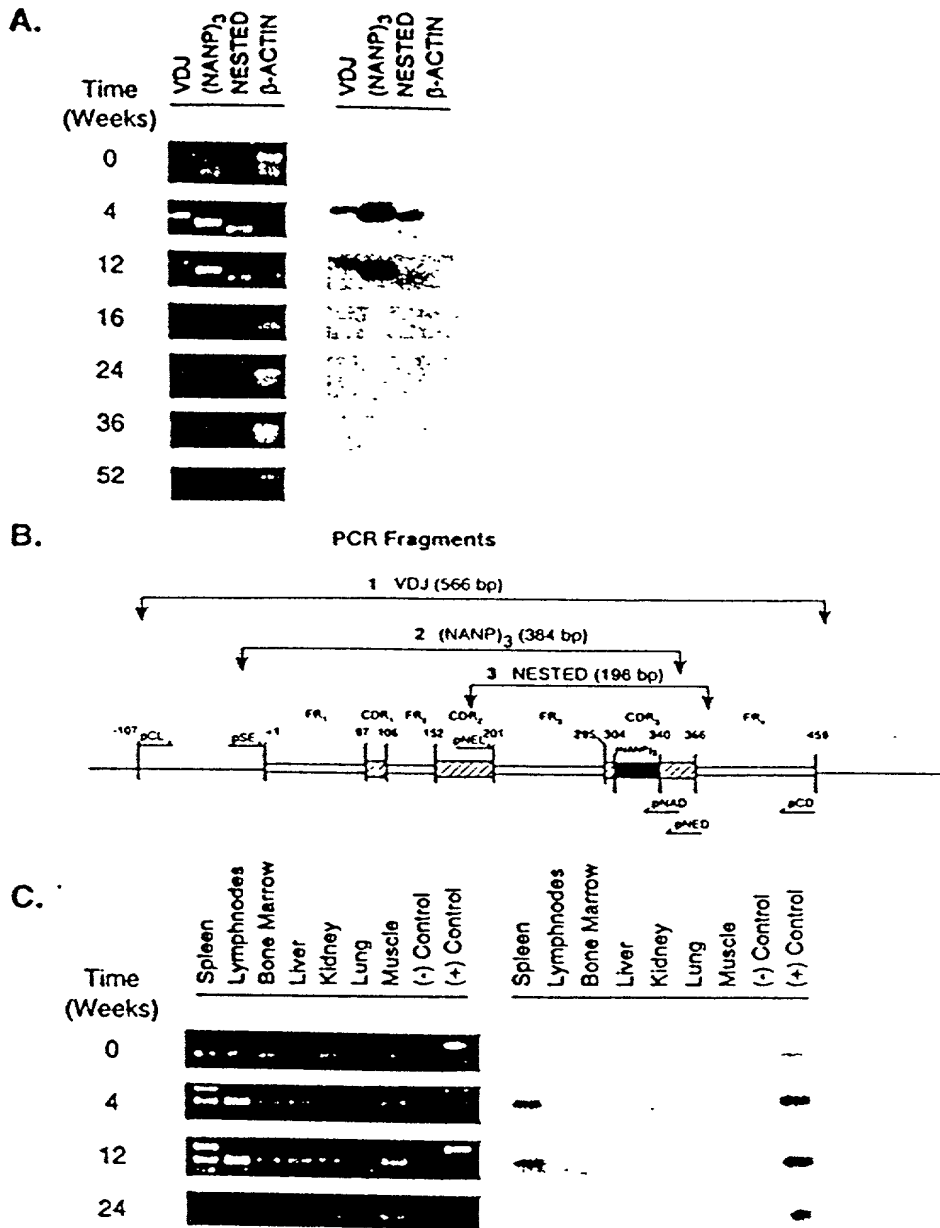


FIGURE 7

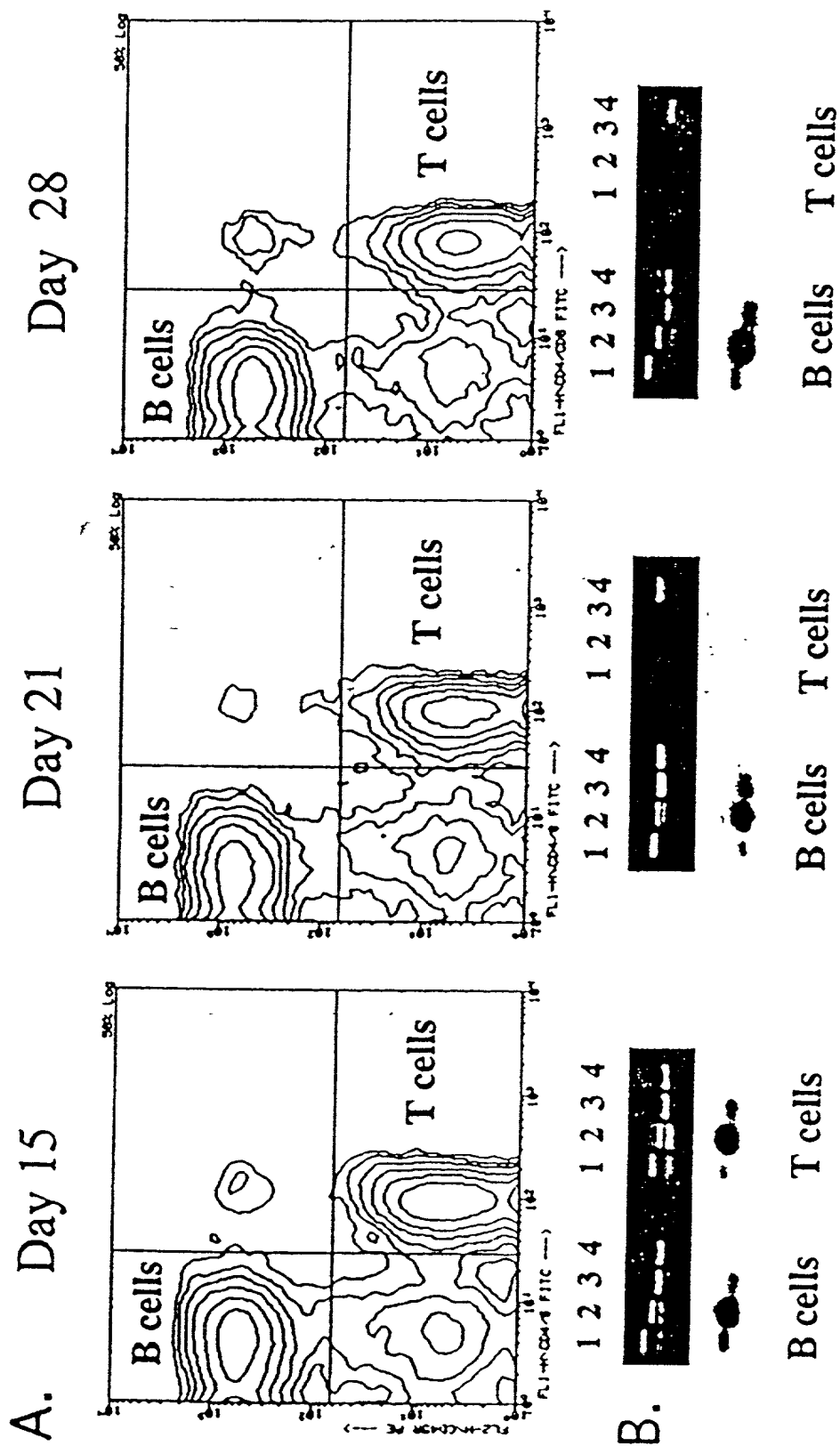


FIGURE 8

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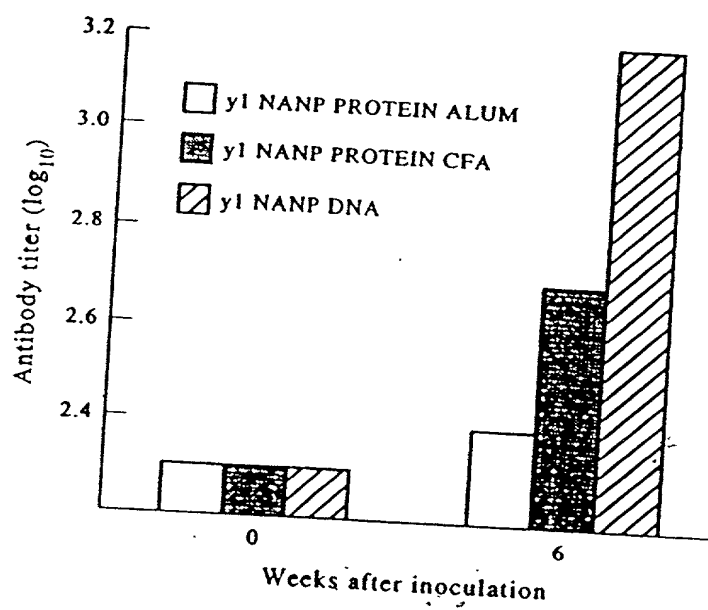


FIGURE 9

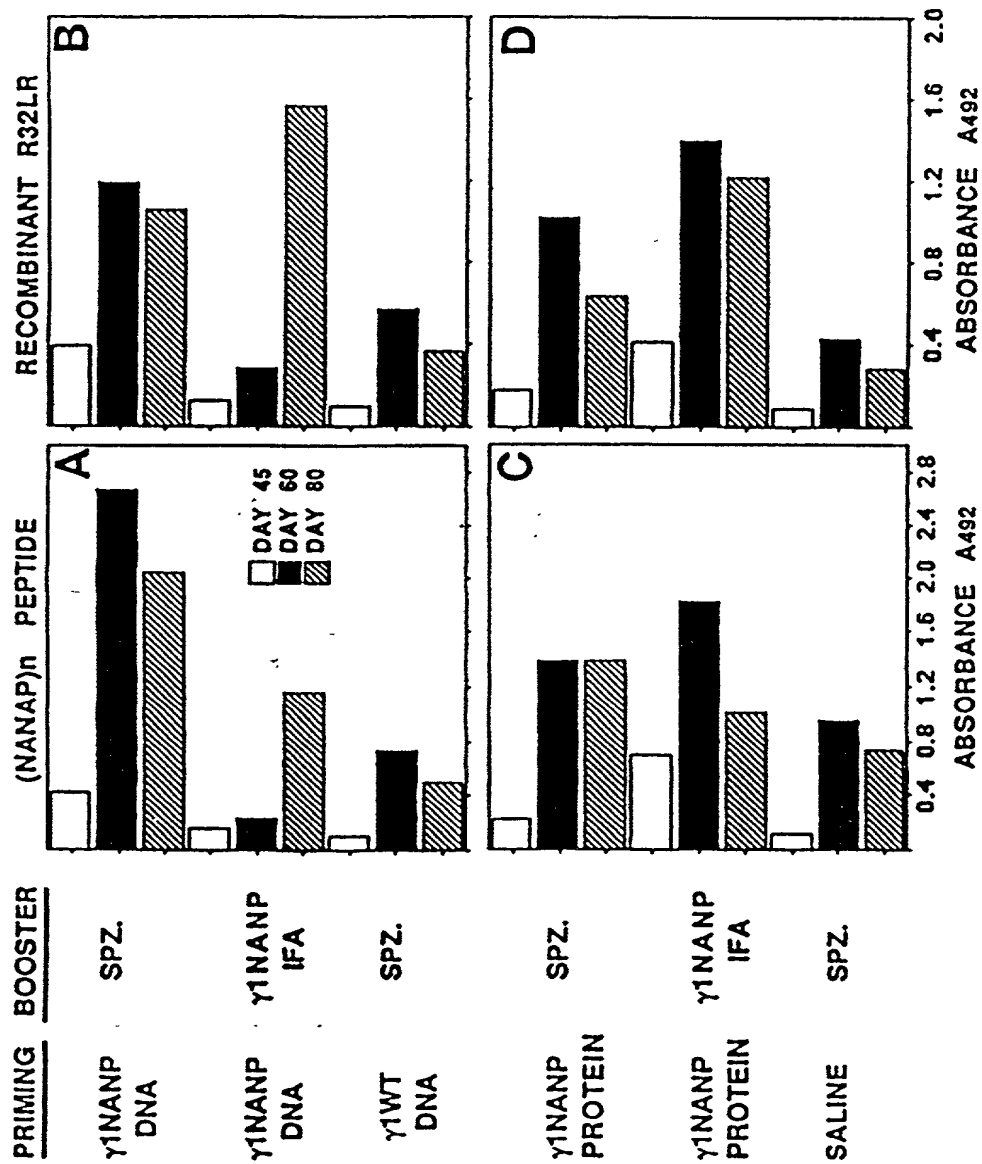


FIGURE 10

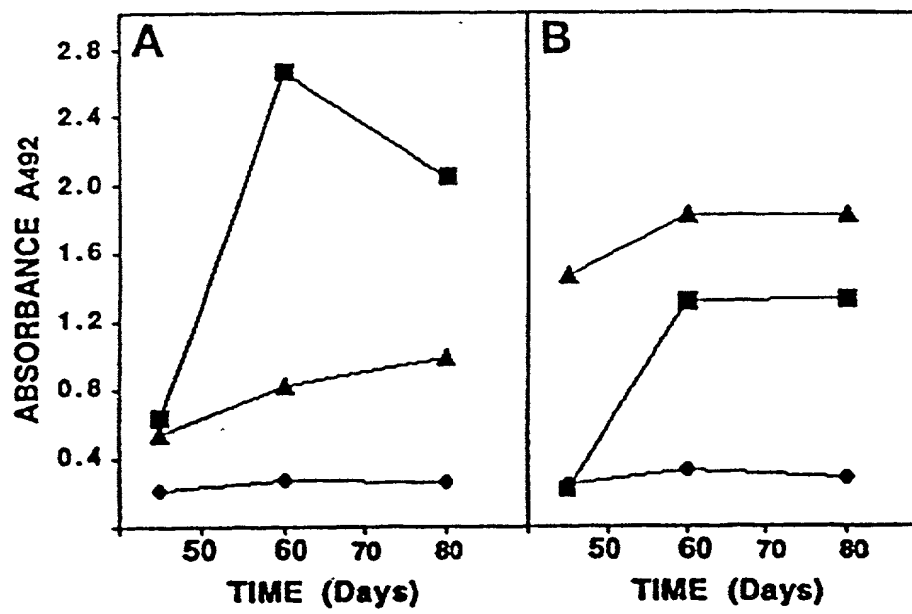


FIGURE 11

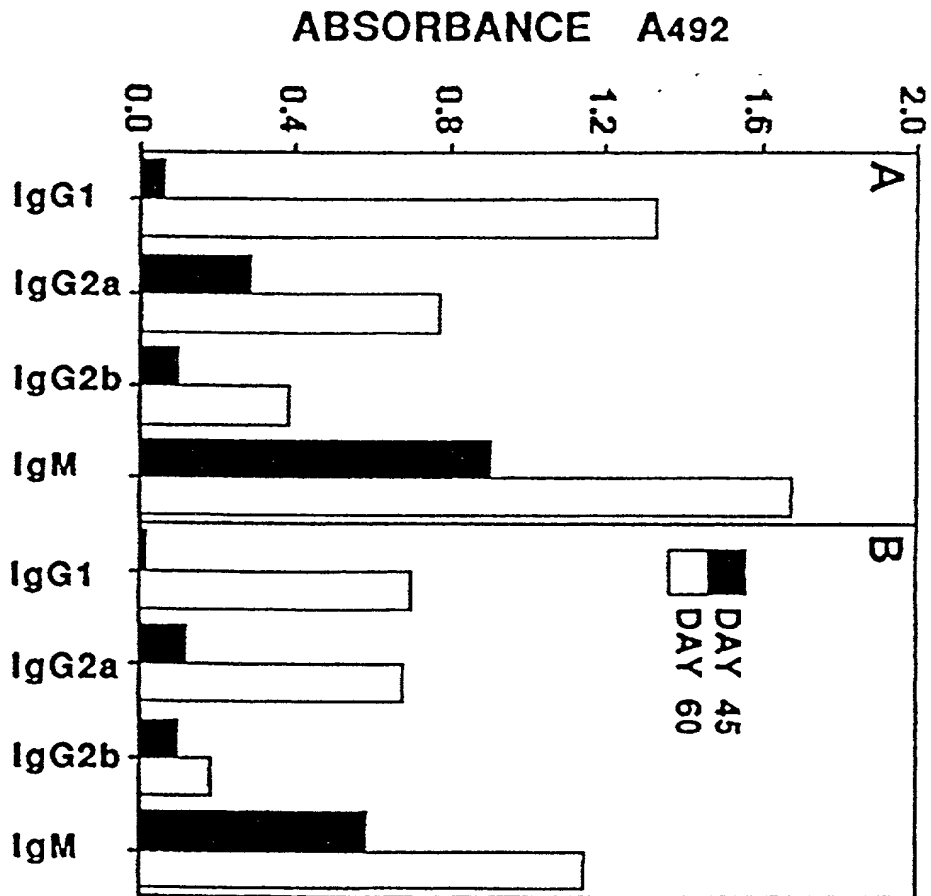


FIGURE 12

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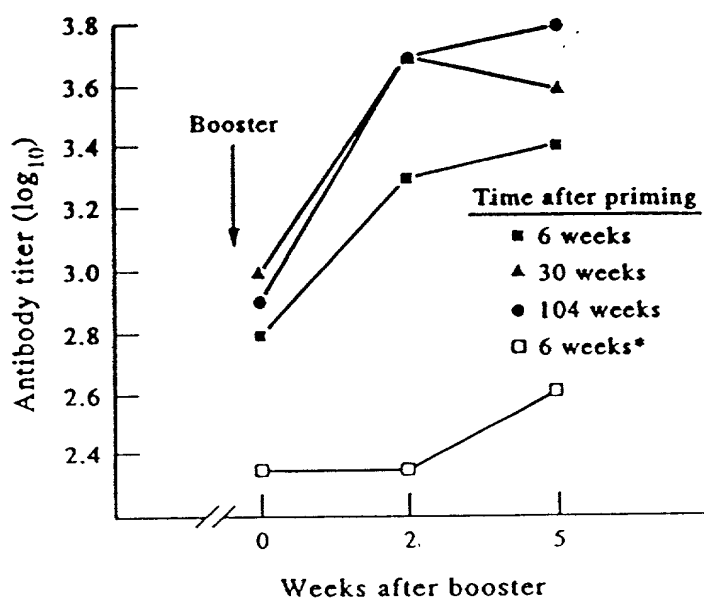


FIGURE 13

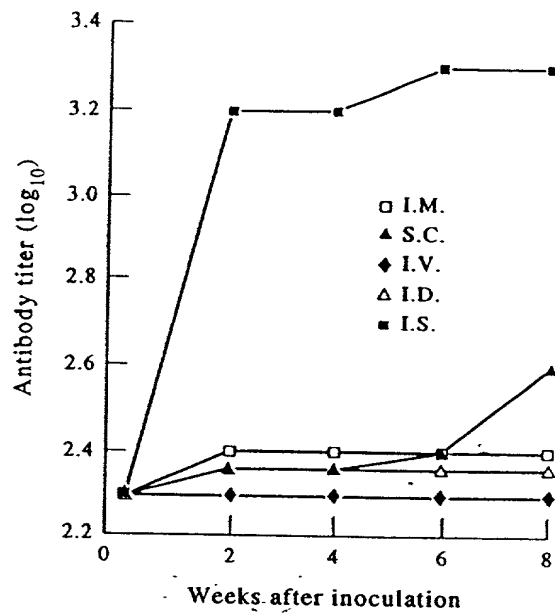


FIGURE 14

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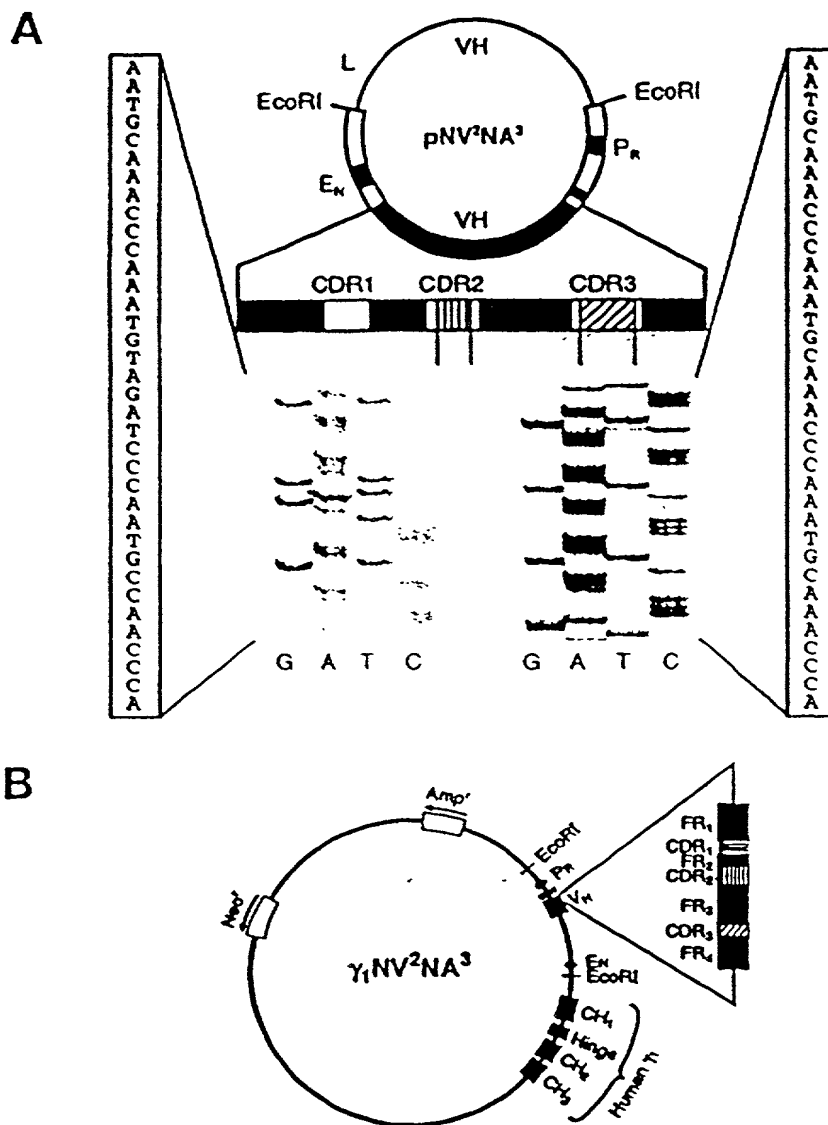


FIGURE 15

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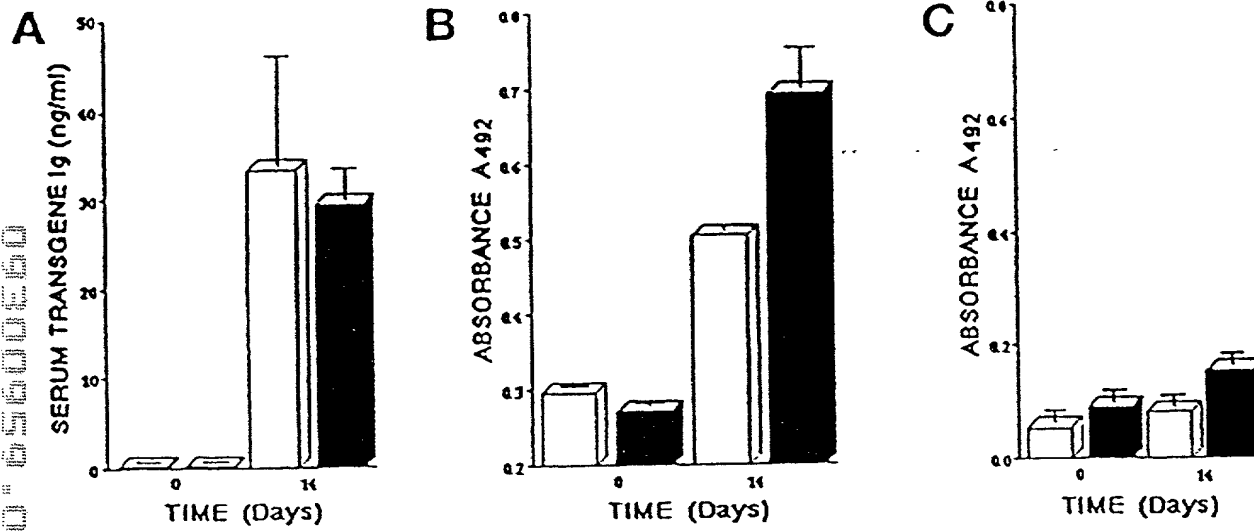


FIGURE 16

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Docket: P-ZA 3519

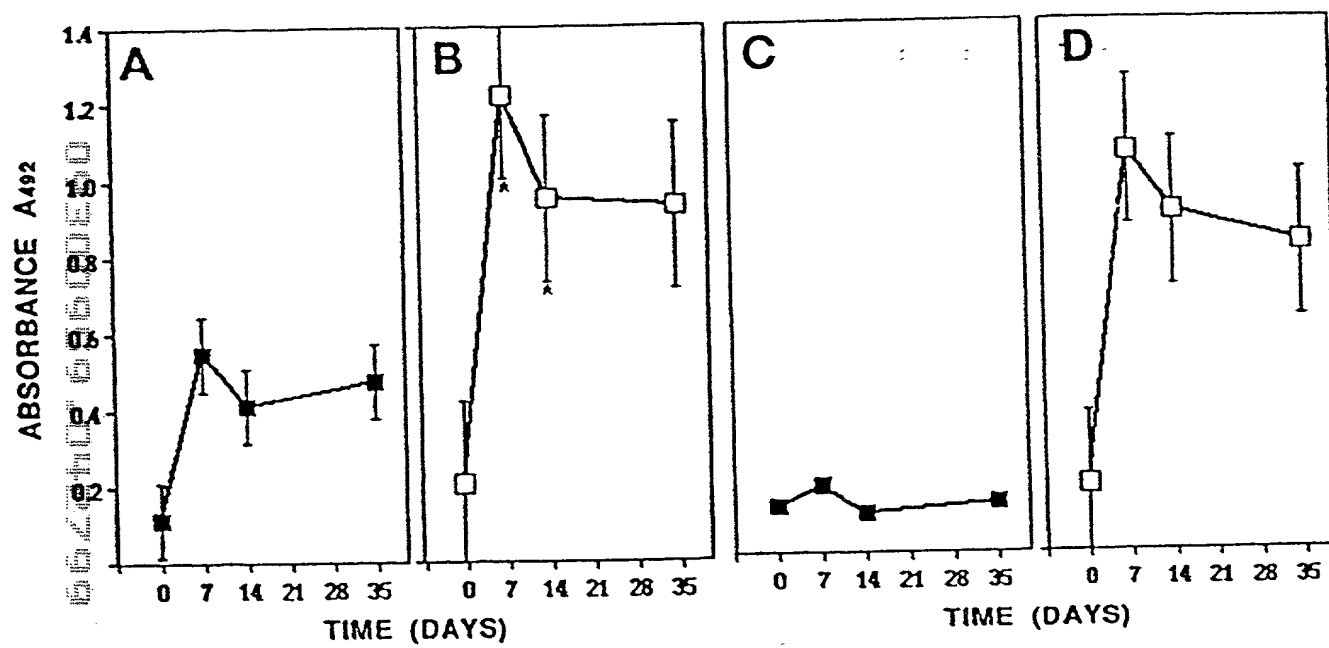


FIGURE 17

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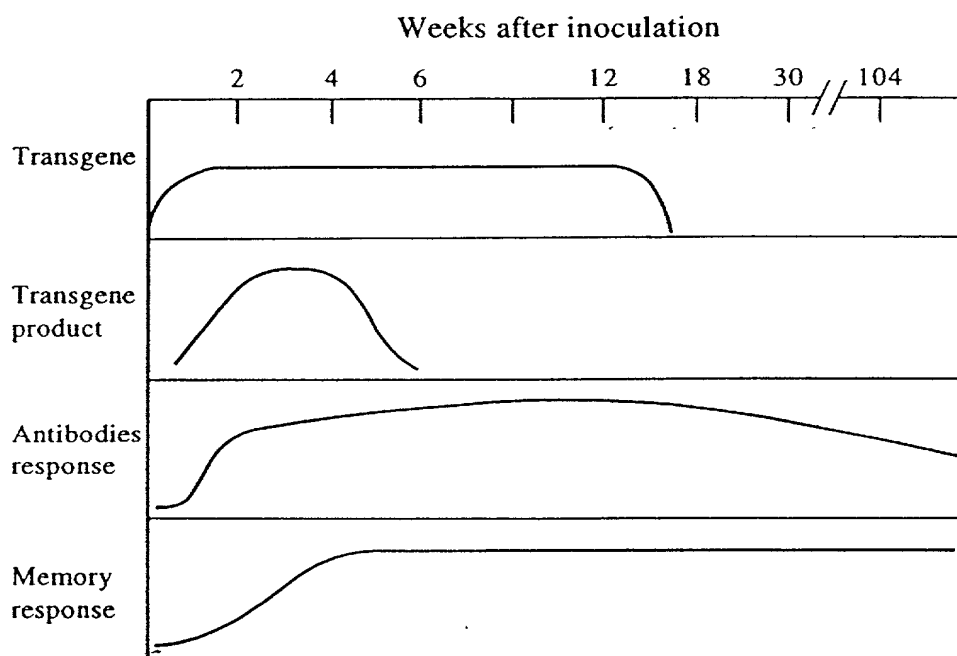


FIGURE 18

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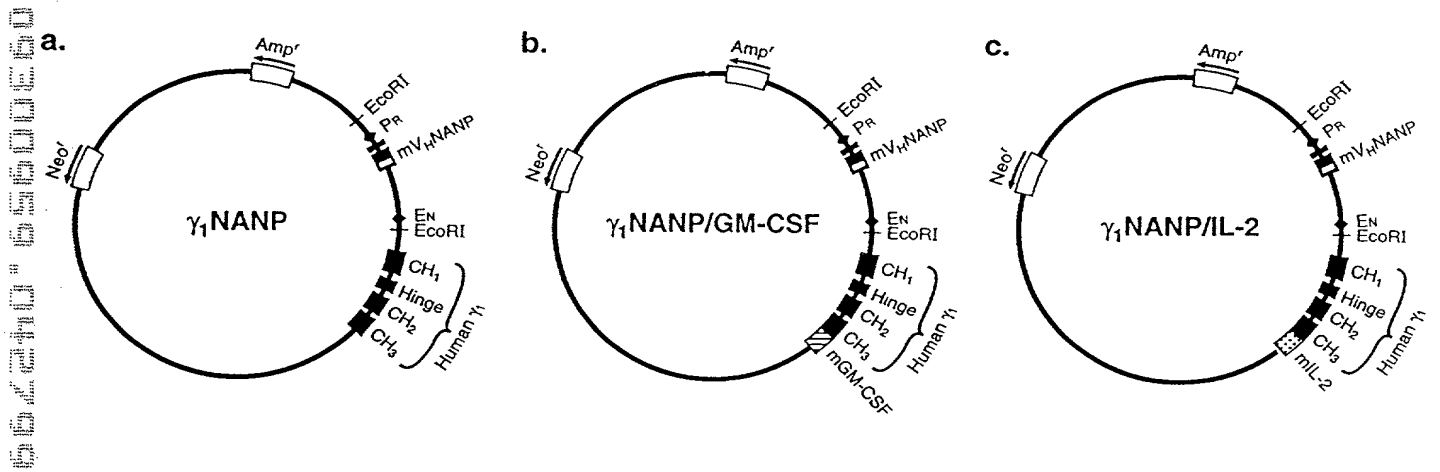


FIGURE 19

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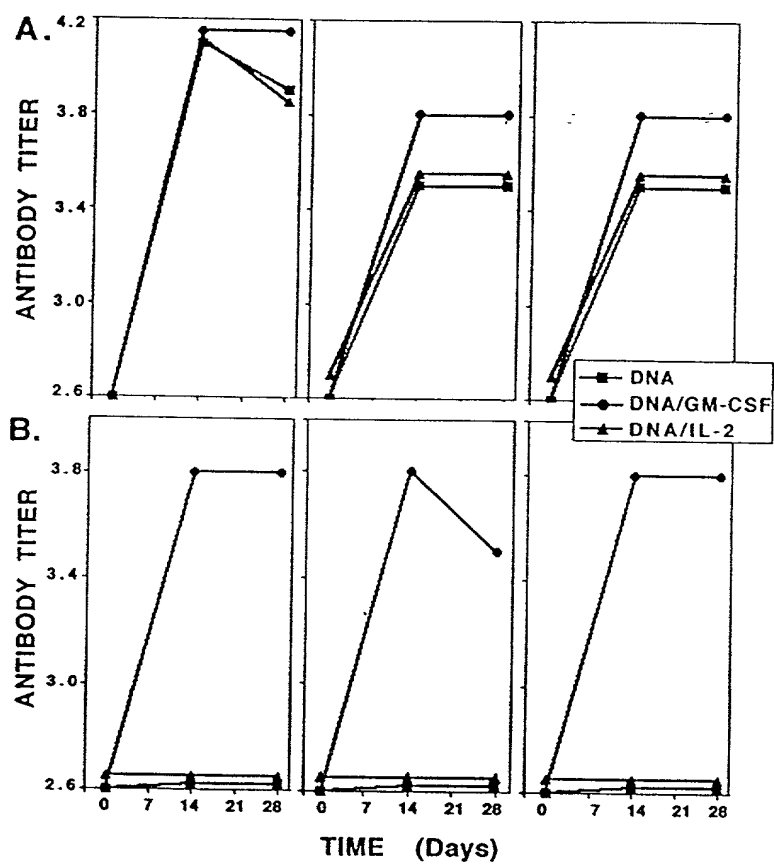


FIGURE 20

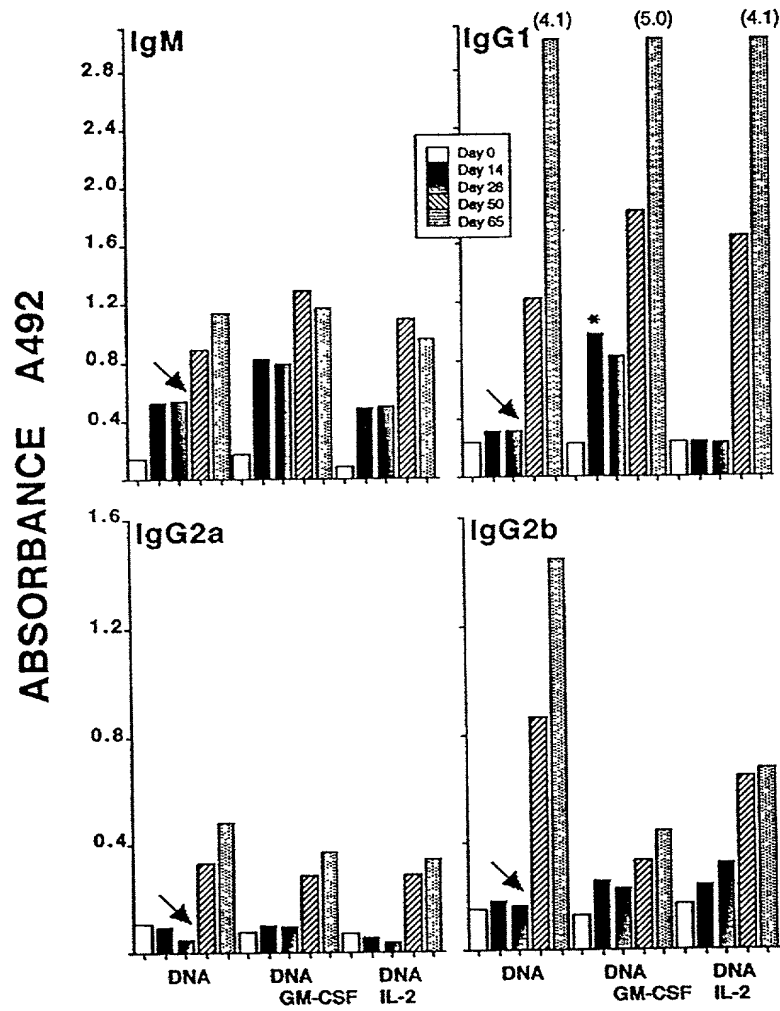


FIGURE 21

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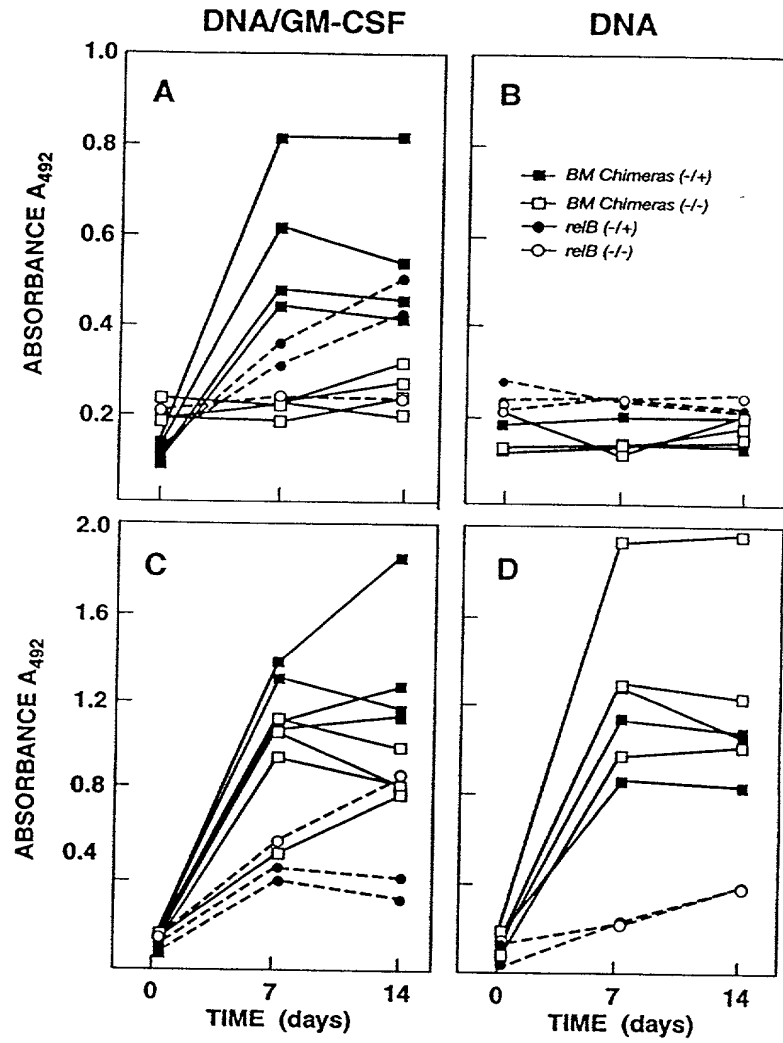


FIGURE 22

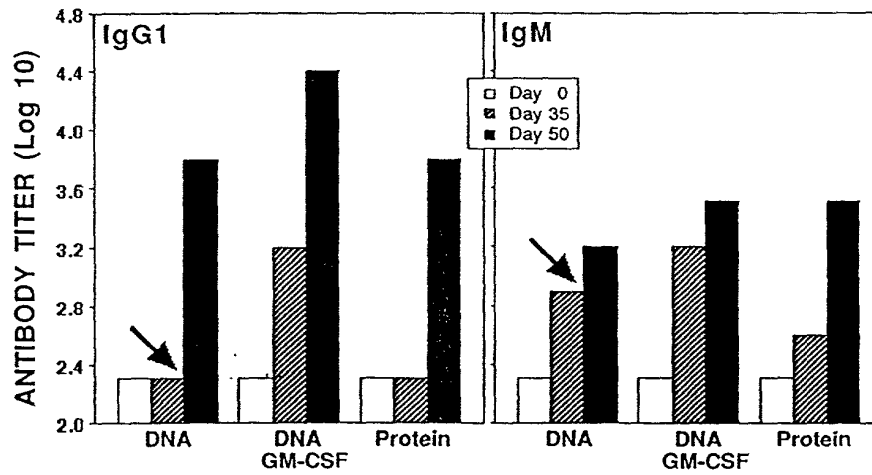


FIGURE 23

Inventor: M. Zanetti

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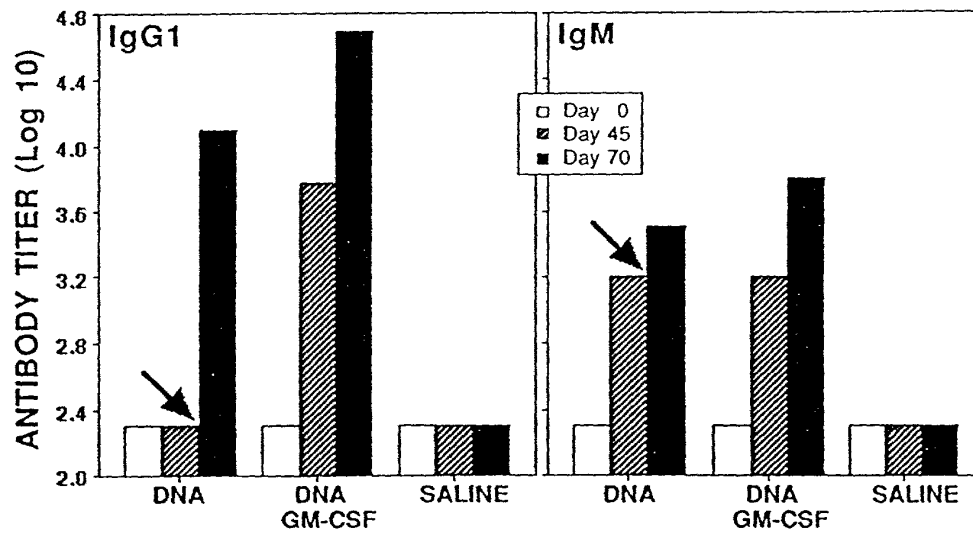


FIGURE 24

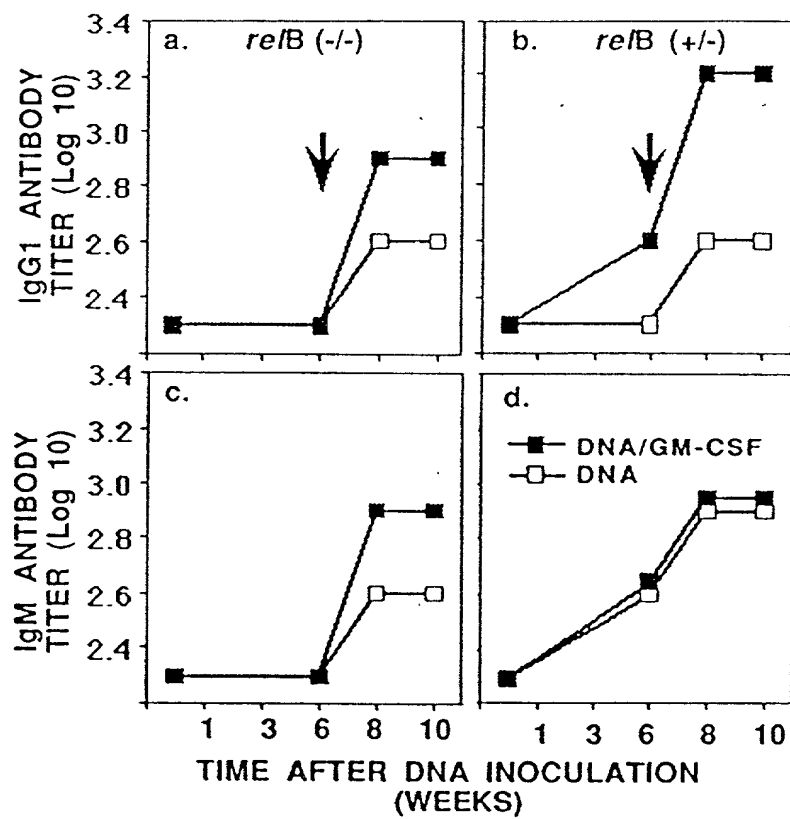


FIGURE 25

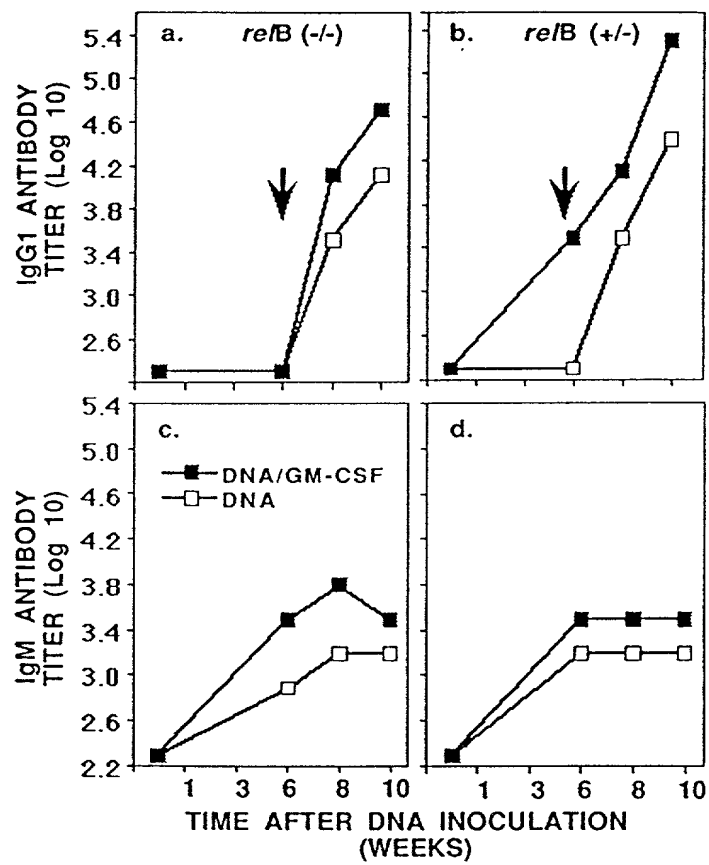


FIGURE 26

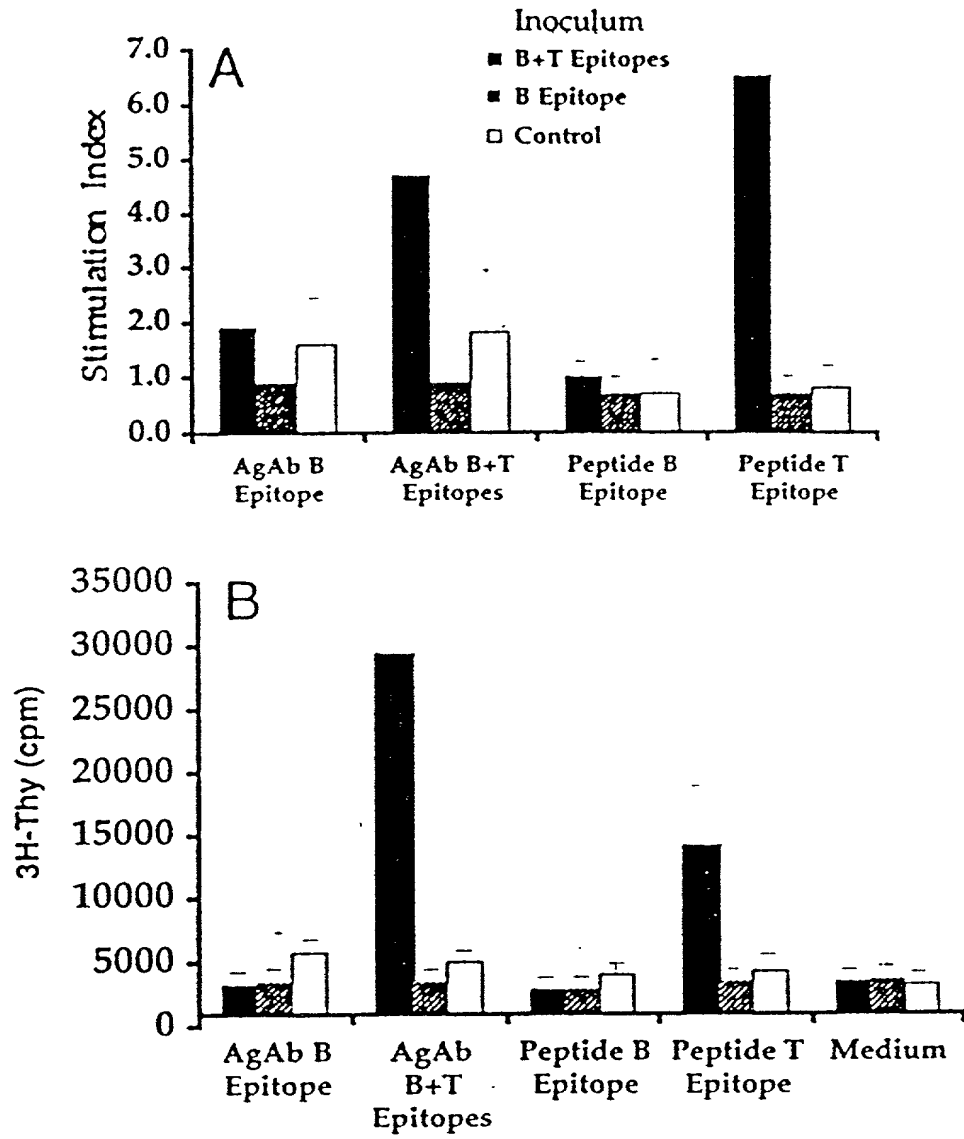


FIGURE 27

Inventor: M. Zanetti

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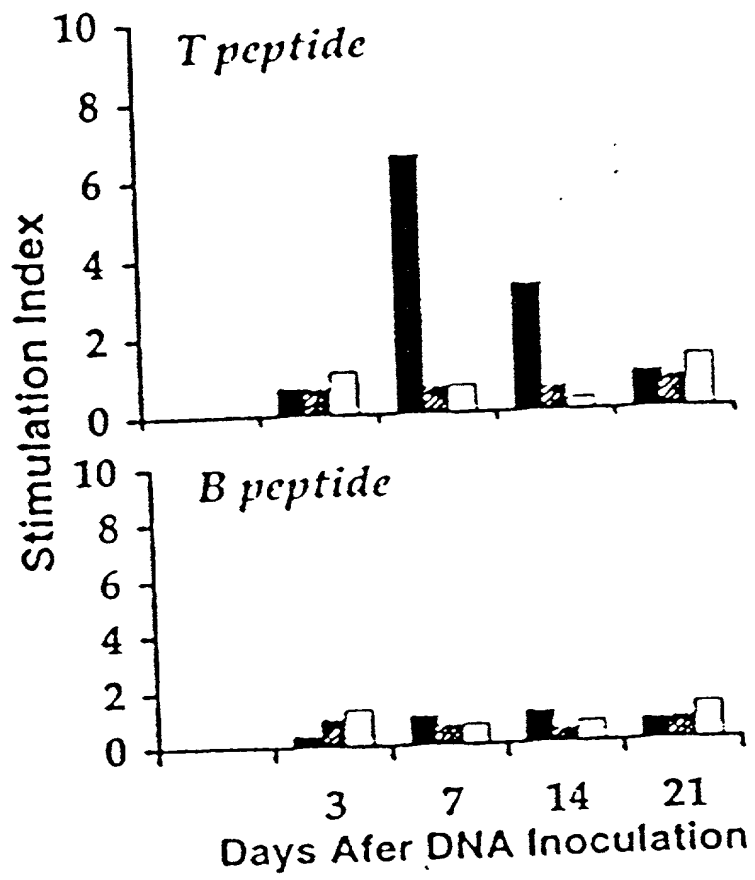


FIGURE 28

Inventor: M. Zanetti

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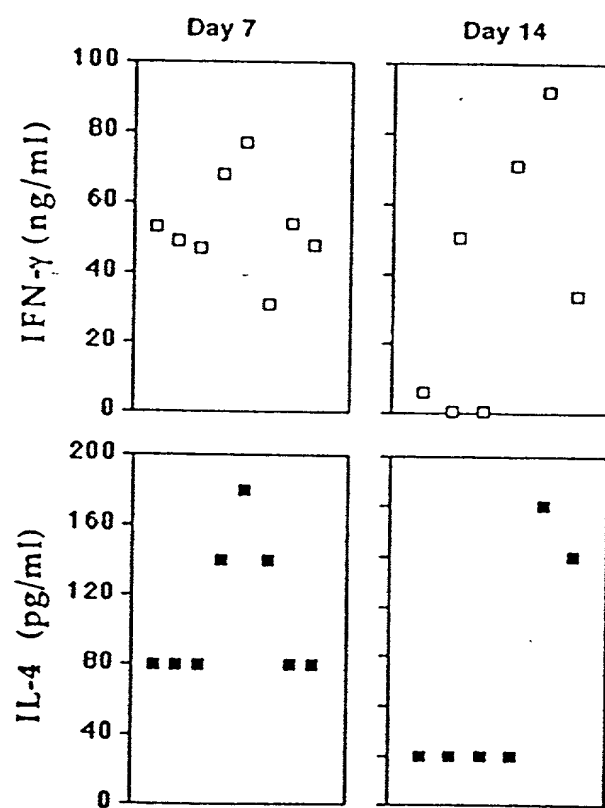


FIGURE 29

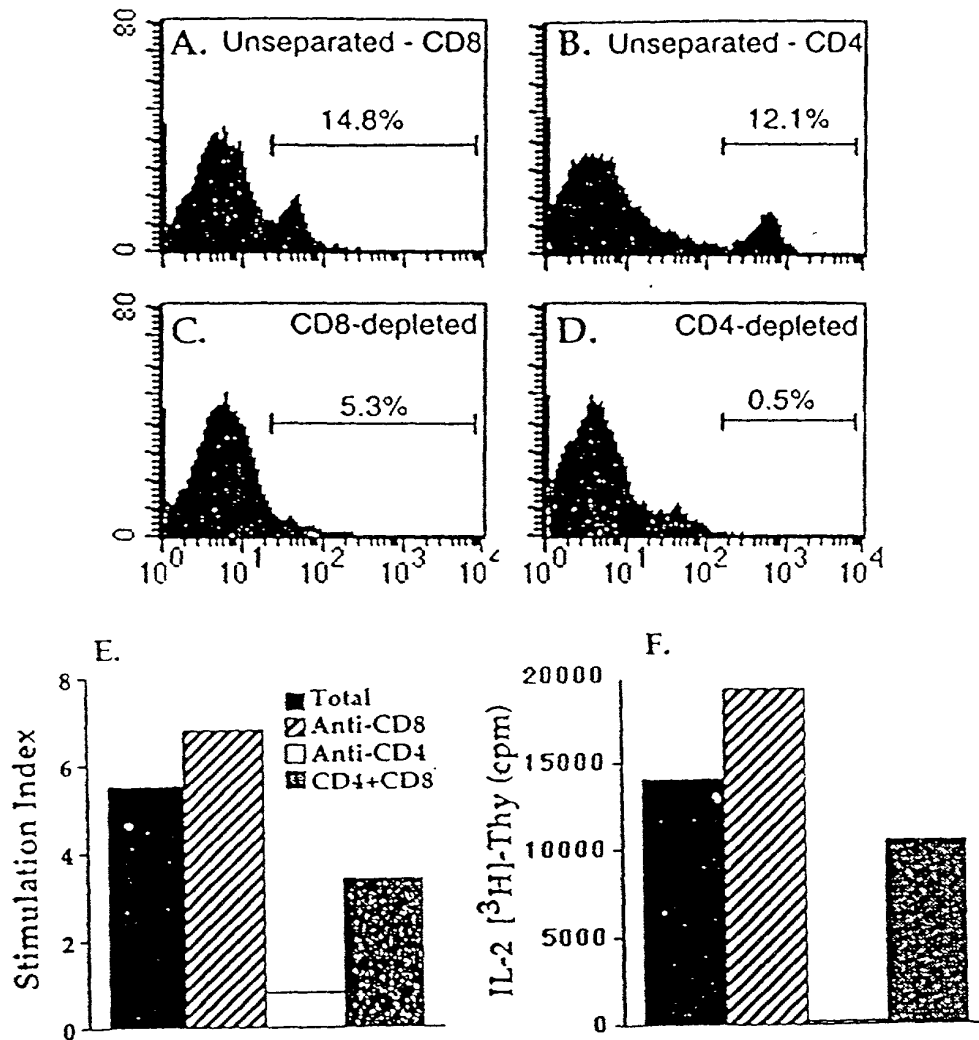
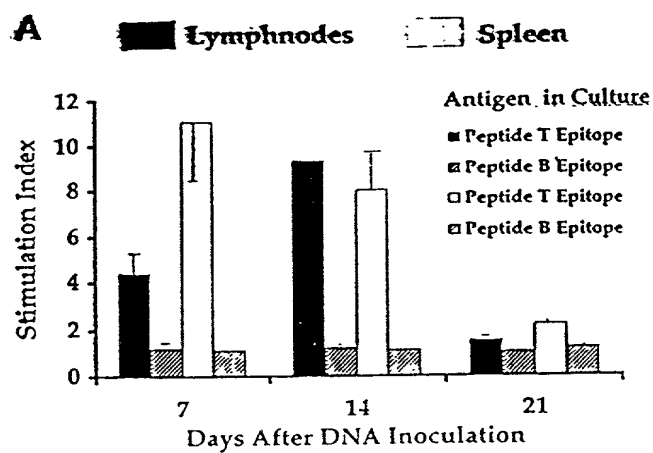


FIGURE 30

09300959 042799
66/240 65600E60



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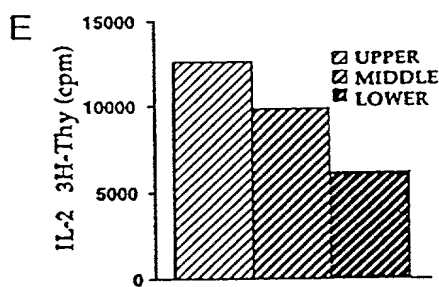
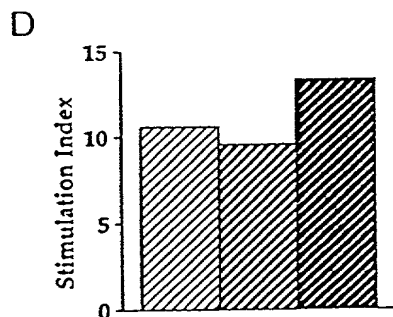
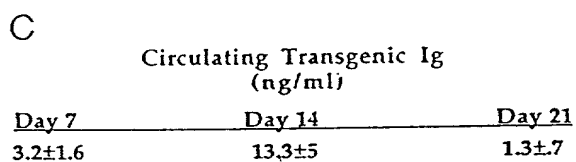
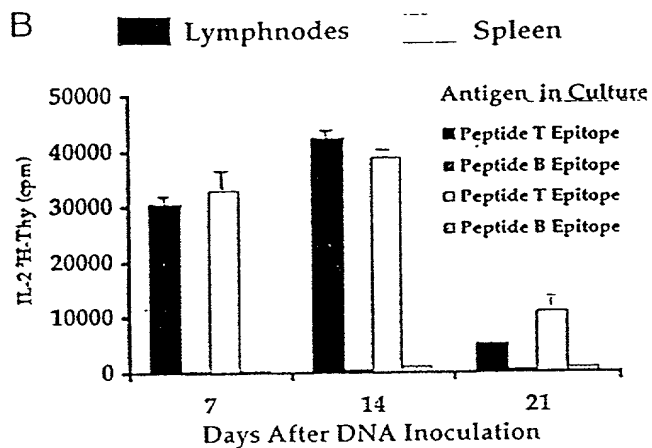
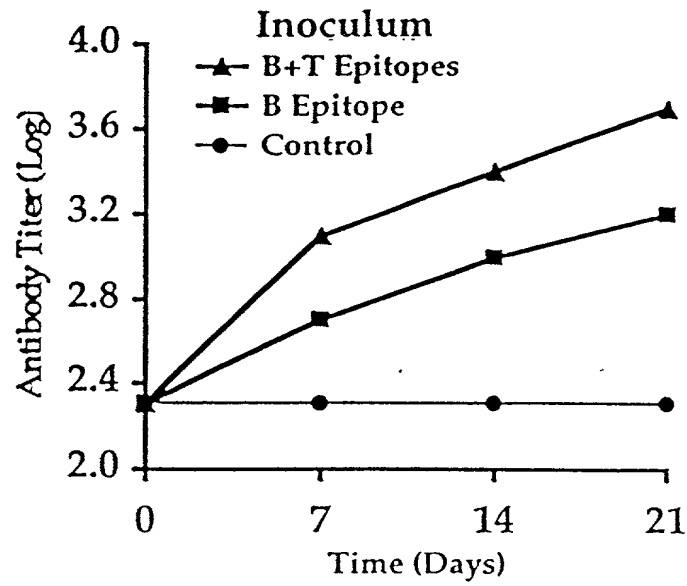
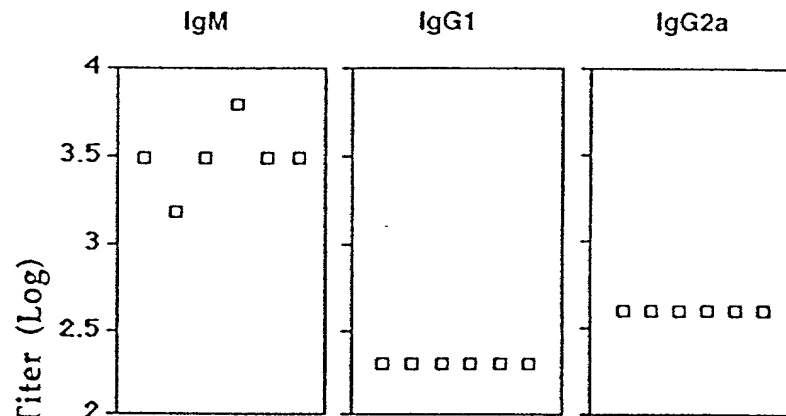


FIGURE 31

A



B



C

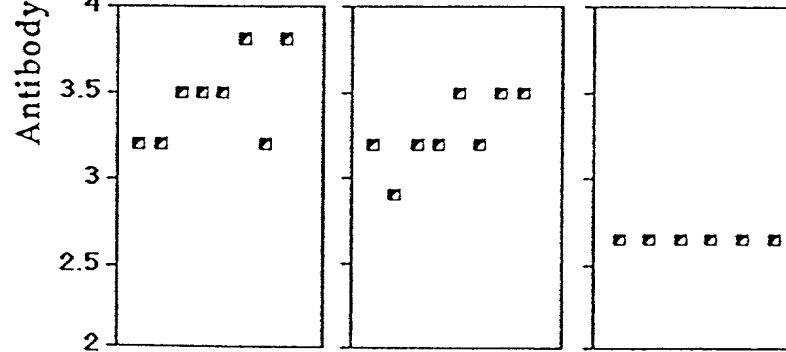


FIGURE 32

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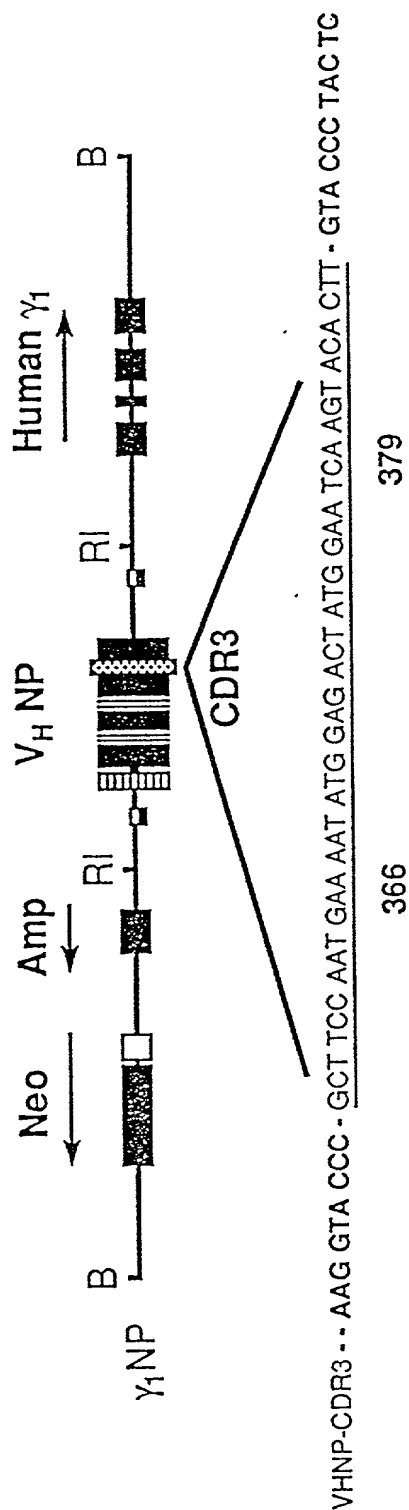


FIGURE 33

Protection Against Infection by Influenza

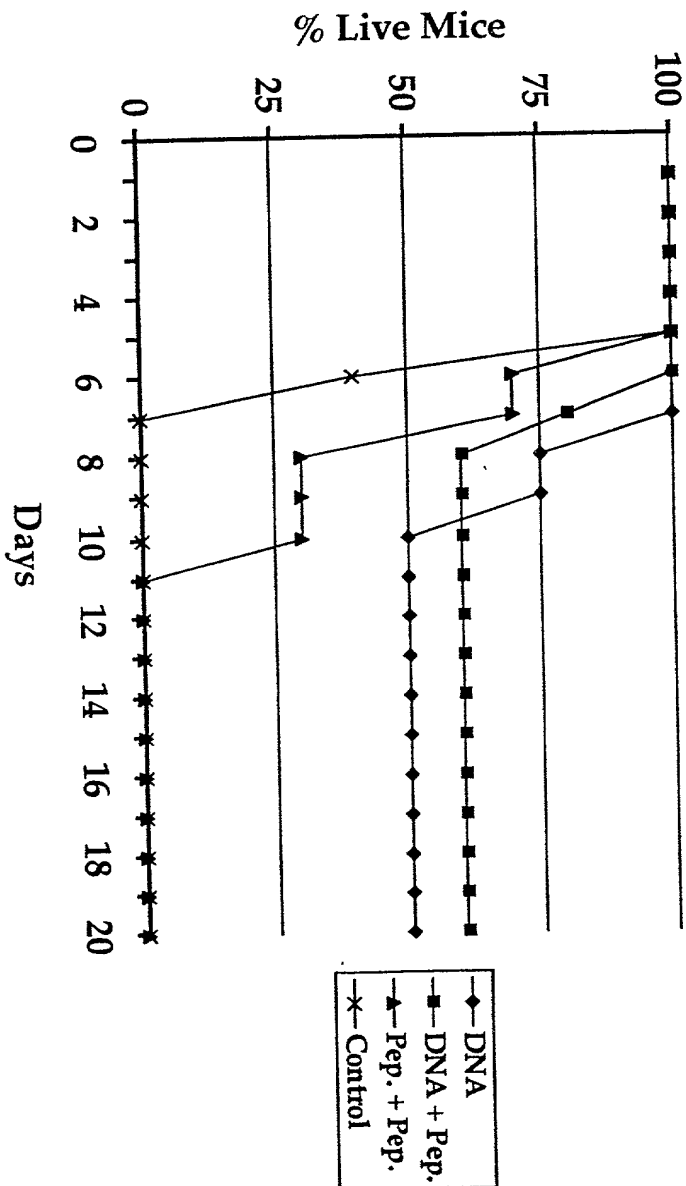


FIGURE 34

09300959 1042799

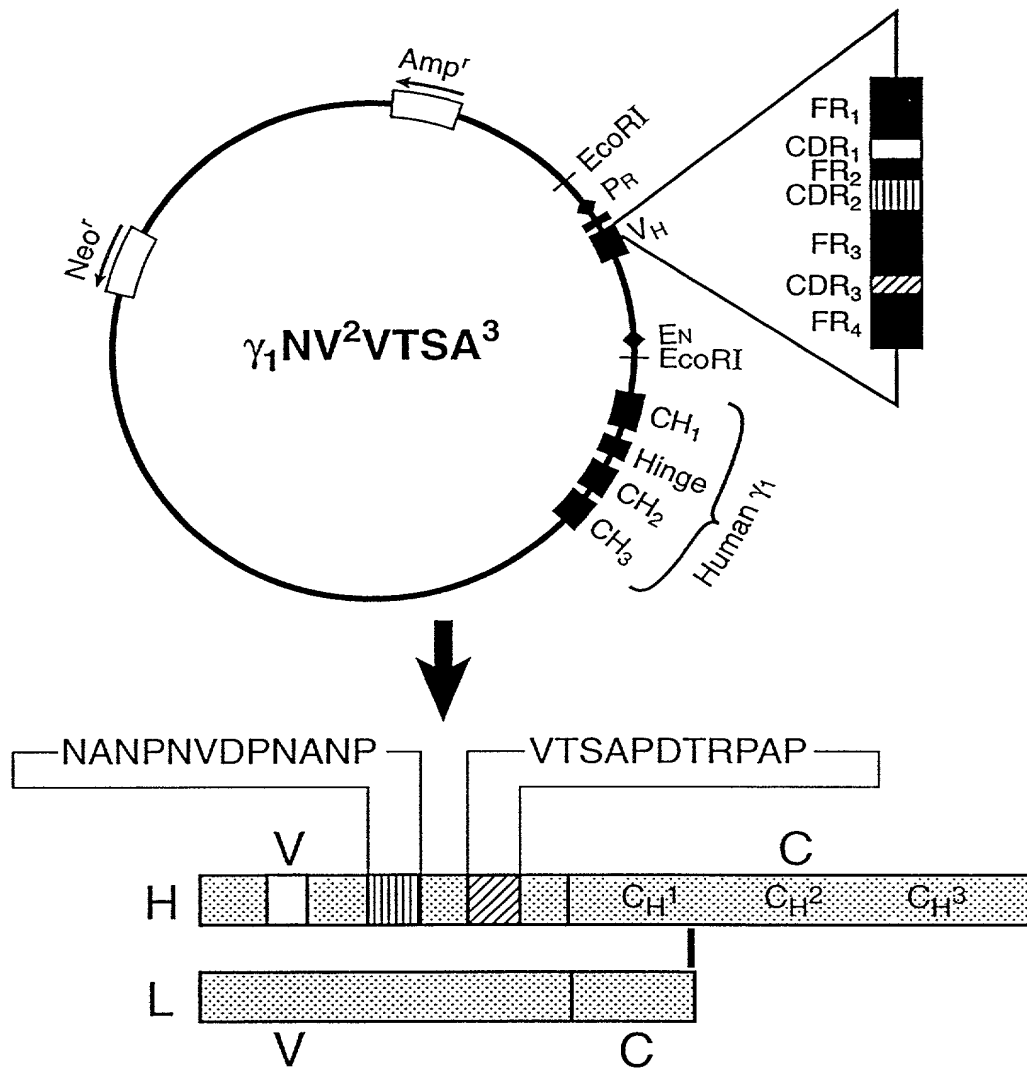


FIGURE 35

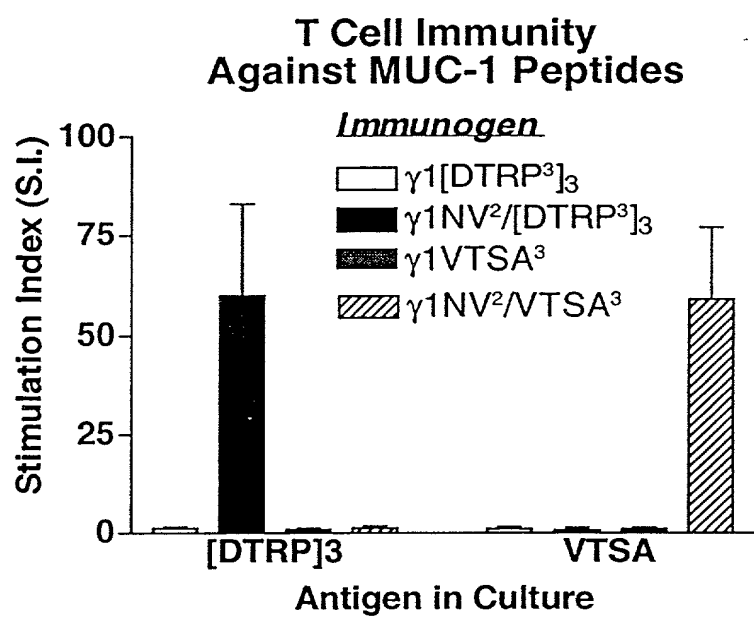
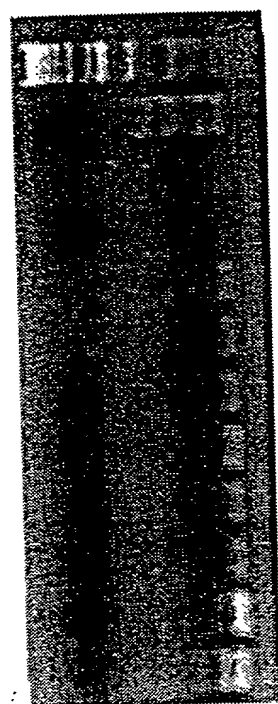


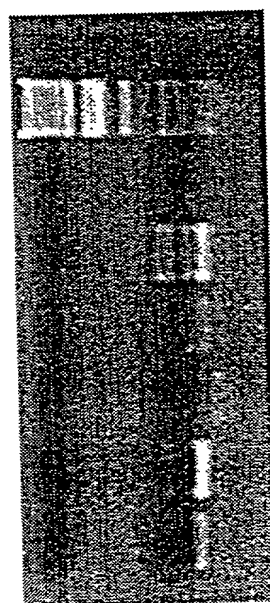
FIGURE 36

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A.



B.

FIGURE 37